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The p53 protein contains several functional domains. We found that activation domain 2 and proline-rich domain are necessary for the apoptotic activity but not cell cycle arrest and form an activation domain for inducing pro-apoptotic genes. We also found that at least two of the three domains, that is, activation domain 1 and 2 and the proline-rich domain, are necessary for cell cycle arrest. Interestingly, we found that deletion of activation domain 1 alleviates the requirement of the C-terminal basic domain for apoptotic activity. Therefore, we generated a small but very potent apoptotic inducer of p53 in MCF7 Breast cancer cells.

To determine the efficacy of chemotherapeutic agents in inducing apoptosis, we found that at a comparable expression level, p73, but not p53, cooperate DNA Damage agents to induce apoptosis in MCF7 cells in a p53-dependent manner.

As an extension of our studies to determine how p53 functions as a tumor suppressor, we identified five novel p53 and p73 target genes, including Dickkopf-1 (Dkk-1), an antagonist of the Wnt oncogenic pathway, and TAP1, a transporter of major histocompatibility class I antigens. This suggests that p53 may suppress transformation by Wnt and play a role in immunosurveillance.

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1. Zhu, J., S. Nozell, J. Wang, J. Jiang, W. Zhou, and **X. Chen.** 2001. ***Oncogene In Press.***
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Introduction

Many studies, including our own, have shown that the p53 sequence-specific DNA binding domain is required for cell cycle arrest and apoptosis (2, 4, 10, 20, 21). The vast majority of naturally occurring tumor-derived p53 mutations are also located in this region (20). In contrast, various functions mapping to the N- and C-terminal domains could be dispensable for one p53 activity but not the other (4, 25). For example, the C-terminal 30 amino acids are dispensable for the transcriptional activity of p53 and cell cycle arrest but are required for p53-dependent apoptosis (5, 13). Therefore, we proposed in this grant to study the regions outside the p53 DNA binding domain, specifically the N- and C-termini of p53. We proposed to use tetracycline inducible expression system to generate MCF7 and H1299 cell lines that inducibly express various defined p53 mutants competent in inducing cell cycle arrest, apoptosis, or both. In addition, by using these cell lines inducibly expressing p53, we proposed to analyze the efficacy of induction of apoptosis by various therapeutic agents and to identify potential mediators for p53-dependent apoptosis.

Body

p73 cooperates with DNA damage to induce apoptosis in MCF7 cells in a p53-dependent manner

p73 is a member of the p53 family (4, 18, 30). Recent experiments have shown that, like p53, p73 can induce both cell cycle arrest and apoptosis (16, 19, 32). Since p53-mediated apoptosis can be augmented by various cancer chemotherapeutic agents, it has been hypothesized that the status of the endogenous p53 gene in tumor cells is a key determinant in the outcome of cancer therapy (5, 23, 29). To determine whether chemotherapeutic agents can affect p73-dependent apoptosis, several cell lines that inducibly express p73 under a tetracycline-regulated promoter were generated. We found that whereas p53-mediated apoptosis was increased, p73-mediated apoptosis was inhibited by several DNA damaging agents in H1299 cells that are p53-null. However, in MCF7 cells that harbor an endogenous wild-type p53 gene, p73-mediated apoptosis was significantly enhanced. Likewise, we found that p73 up-regulation of cellular target genes, such as p21 and 14-3-3σ, was increased in MCF7 cells but inhibited in H1299 cells when treated with the DNA damaging agent camptothecin. Furthermore, in MCF7E6 cells that ectopically express the human papillomavirus E6 oncogene and are functionally p53-null, the cooperative induction of apoptosis and cellular target genes by DNA damage and p73 was abrogated or severely diminished. Taken together, these results suggest that p53 and p73 are differentially modified by DNA damaging agents in H1299 cells and a functional interaction between p53 and p73 in MCF7 cells leads to enhanced induction of apoptosis. For detail, please see the attached manuscript #1.

Identification and characterization of p53 functional domains that are necessary for inducing cell cycle arrest and apoptosis.

The ability of p53 to induce apoptosis requires its sequence-specific DNA binding activity (10, 20, 21); however, the transactivation-deficient p53(gln22-ser23), designated p53(AD1$^-$), can still induce apoptosis (5, 13). Previously, we showed that the region between residues 22 and 97 in p53 is necessary for apoptosis (5). In an effort to more precisely map this domain within the N-terminus, we found that deletion of the N-terminal 23 amino acids compromises, but does not abolish, p53 induction of apoptosis. Surprisingly, p53(ΔAD1), which lacks the N-terminal 42 amino acids and the previously defined activation domain, retains the ability to induce apoptosis to an even higher level than wild-type p53. However, a more extensive deletion, which eliminates the N-terminal 63 amino acids (ΔAD1ΔAD2), renders p53 completely inert in mediating apoptosis. In addition, we found that both p53(ΔAD1) and p53(AD1$^-$) can activate a subset of target genes. Furthermore, we showed that residues 53 and 54 are critical for the apoptotic and transcriptional activities of both p53(ΔAD1) and p53(AD1$^-$). Taken together, these data suggest that within residues 43 to 63 lies an apoptotic domain as well as another transcriptional activation domain. We therefore postulate that the apoptotic activity in p53(AD1$^-$) and p53(ΔAD1) is still transcription-dependent. For detail, please see the attached manuscript #2.

The proline-rich domain is located within residues 60-90, which comprise five PXXP motifs (where P represents proline and X is any amino acid). The proline-rich domain has been shown to be necessary for efficient growth suppression (28). To further delineate the potential role of the proline-rich domain in transactivation, cell cycle arrest, and apoptosis, we generated several cell lines that inducibly express various p53 mutants. We found that p53(ΔPRD), which lacks all five PXXP motifs, is capable of inducing cell cycle arrest but not apoptosis, while p53(AD1$^-$ΔPRD), which contains a double point mutation in the activation domain as well as deletion of the proline-rich domain, completely loses its activity. However, p53(Δ74-91), which contains only one PXXP motif at its N-terminus, is not only capable of inducing cell cycle arrest but also retains partial apoptotic activity. We also found that deletion of the proline-rich region has no or very mild effects on activation of several transiently transfected p53 target gene promoters, i.e., the p21, MDM2, BAX, and GADD45 promoters. However, such deletion differentially affects p53 induction of endogenous target genes. We found that induction of p21, MDM2, BTG2, p85, PIG3, PIG6, and PIG11 was reduced or abrogated, but induction of BAX, KILLER/DR5, PIG2, PIG7, and PIG8 was not substantially affected. Interestingly, induction of GADD45 was enhanced. These results suggest that the proline-rich domain may play a role in chromatin remodeling, which counteracts chromatin-mediated repression for some of the endogenous p53 target genes. For detail, please see the attached manuscript #3.

The above results and our earlier observation (5) indicate that activation domain 2, the proline-rich domain, and the C-terminal basic domain within residues 364-393 are required for apoptotic activity. To further define the essential domains necessary for inducing apoptosis, we have generated several additional p53 mutants. We found that an activation domain 2 mutation at residues 53-54 (AD2$^-$) abrogates apoptotic activity but has no significant effect on cell cycle arrest. We also found that p53(ΔAD2), which lacks activation domain 2, is inert in inducing apoptosis. p53(AD2$^-$ΔBD), which is defective in activation domain 2 and lacks the C-terminal basic domain, p53(ΔAD2ΔBD), which lacks both activation domain 2 and the C-terminal basic domain, and p53(ΔPRDΔBD), which lacks both the proline-rich domain and the C-terminal basic

domain, are also inert in inducing apoptosis. All four mutants are still active in inducing cell cycle arrest, albeit to a lesser extent than wild-type p53. Interestingly, we found that deletion of the N-terminal activation domain 1 alleviates the requirement of the C-terminal basic domain for apoptotic activity. Thus, we have generated a small but potent p53 molecule p53(ΔAD1ΔBD). Furthermore, we found that at least two of the three domains, that is, activation domain 1, activation domain 2, and the proline-rich domain, are required for inducing cell cycle arrest. For detail, please see the attached manuscript #4.

Identification and characterization of p53 target genes (p73, MCG10, Dkk-1, TAP1, and AQP3).

Regulation of the p73 gene. Recent studies have shown that DNA damage can stabilize p73 protein and enhance p73-mediated apoptosis in a c-Abl dependent manner (1, 12, 31). To determine what regulates p73 transcription, we analyzed the expression of p73 in several cell lines following genotoxic stresses. We found that p73 is induced in some cell lines when treated with chemotherapeutic agents. We also found that p53 and p73, but not mutants p53(R249S) and p73β292, directly induce the expression of the p73 gene. In addition, we found one potential p53-binding site in the promoter of the p73 gene, which is responsive to p53, p73, and DNA damage. Taken together, these data suggest that p73 is transcriptionally autoregulated and regulated by DNA damage and p53. Since both p53 and p73 proteins can be stabilized by DNA damage, we propose that in normal cells, DNA damage stabilizes and activates p53 and p73, and the resulting activated p53 and p73 proteins can each induce the expression of cellular target genes, including the p73 gene itself. For detail, please see the attached manuscript #5.

Identification of MCG10 as a p53 target gene. MCG10, a novel p53 target gene, was identified in a cDNA subtraction assay with mRNA isolated from a p53-producing cell line. MCG10 can be induced by wild type, but not mutant, p53 and by DNA damage via two p53 response elements in the promoter of the MCG10 gene. The MCG10 gene contains 10 exons and is located on chromosome 3p21, a region highly susceptible to aberrant chromosomal rearrangements and deletions in human neoplasias. The MCG10 gene locus encodes at least two alternatively spliced transcripts, MCG10 and MCG10as. The MCG10 and MCG10as proteins contain two domains homologous to the heterogeneous nuclear ribonucleoprotein (hnRNP) K homology (KH) domain (26). By generating cell lines that inducibly express either wild-type or various mutated forms of MCG10 and MCG10as, we found that MCG10 and MCG10as can suppress cell proliferation by inducing apoptosis and cell cycle arrest in G2-M. In addition, we found that MCG10 and MCG10as, through their KH domains, can bind poly(C) and that their RNA binding activity is necessary for inducing apoptosis and cell cycle arrest. Furthermore, we found that the level of the poly(C) binding MCG10 protein is increased in cells treated with DNA-damaging agent camptothecin in a p53-dependent manner. For detail, please see the attached manuscript #6.

Identification of Dickkopf-1 as a p53 target gene. Dickkopf-1 (Dkk-1), a secreted glycoprotein, has been found to be necessary and sufficient for inducing amphibian head formation (11). Interestingly, the mechanism by which Dkk-1 does this is the ability of Dkk-1 to antagonize the Wnt signaling pathway (11). Wnt, itself a proto-oncoprotein, can promote cell

proliferation and transformation when mutated or overexpressed, leading to tumor formation (3). Additionally, loss of p53 function accelerates mammary tumorigenesis by Wnt (9, 14). In this study, we found that Dkk-1 is induced by wild type p53 but not mutant p53(R249S). In addition, DNA damage upregulates Dkk-1 in cell lines that harbor an endogenous wild-type p53 gene but not in cell lines that are p53-null or harbor an endogenous mutant p53 gene. We also found a potential p53 response element located approximately 2,100 nucleotides upstream of the Dkk-1 transcription start site, and we showed that p53 binds specifically to this element both *in vitro* and *in vivo*. Furthermore, we have established several cell lines derived from H1299 and U118 glioma cells that inducibly express Dkk-1. We found that Dkk-1 has no effect on proliferation of cells that are not transformed by Wnt. Taken together, we found that Dkk-1 may mediate p53 tumor suppression by antagonizing the Wnt signaling pathway. For detail, please see the attached manuscript #7.

The role of TAP1 in p53 tumor suppression. The transporter associated with antigen processing (TAP) 1 protein is required for the major histocompatibility complex (MHC) class I antigen presentation pathway, which plays a key role in host tumor surveillance (24). Since more than 50% of tumors have a dysfunctional p53 (20), evasion of tumor surveillance by tumor cells may be linked to loss of p53 function. We found that TAP1 is strongly induced by p53, p73, and DNA-damaging agents through a p53 response element. We also found that p73 can cooperate with p53 to induce TAP1. Furthermore, we found that by inducing TAP1, p53 enhances the transport of MHC class I peptides and expression of surface MHC-peptide complexes. Thus, we found that tumor surveillance may be one mechanism by which p53 functions as a tumor suppressor. For detail, please see the attached manuscript #8.

Aquaporin 3, a glycerol and water transporter, is regulated by p73 of the p53 family. p73, a member of the p53 family, has been shown to exhibit similar biochemical activities to that of p53 ((18, 30); please also see the attached manuscript #9). However, in contrast to p53, p73 is rarely mutated in human tumors and p73 mutant mice develop neurological, pheromonal, and inflammatory defects, but not spontaneous tumors. Furthermore, p73 mutant mice are deficient in the physiological control of cerebral spinal fluid. To determine what mediates these p73 activities, cDNA subtraction assay was performed to identify cellular genes that are regulated by p73. We found that aquaporin 3 (AQP3), a glycerol and water transporter, is regulated by p73. In addition, we identified a potential p53 response element in the promoter of the AQP3 gene, which is responsive to p73. This suggests that AQP3 may mediate the activity of p73 in maintaining cerebral spinal fluid dynamics. For detail, please see the attached manuscript #10.

p63 and p73 differentially regulate p53 target genes.

To further characterize the role of p63 and p73 in growth suppression, we established several groups of cell lines that inducibly express p63 or p73. We found that p63 and p73 can induce both cell cycle arrest and apoptosis. We also found that p63 and p73 can activate some but not all of the previously identified p53 target genes. Furthermore, we found that the transcriptional activities of p53, p63, and p73 to induce their common cellular target genes differ among one another. These results suggest that p63 and p73 are both similar to and different from

p53 in their signaling pathways leading to growth suppression. The results for p73 were recently published in Cancer Research (please see attached manuscript #11), and the results for p63 were recently published in Oncogene (please see the attached manuscript #12).

Work accomplished in relation to the Statement of Work

Tasks 1-2: Generation and characterization of breast cancer cell lines expressing p53. Using a tetracycline inducible system, we have generated and analyzed a number of MCF7 breast carcinoma cell lines and H1299 lung carcinoma cell lines that inducibly express wild type p53, p63, p73, or various mutated forms of the p53 family members (for detail, please see manuscripts # 1, 2, 3, 4, 11, and 12).

Tasks 3: Efficacy of chemotherapeutic agents to induce apoptosis in cell lines expressing p53. We found that DNA damage enhances p73-dependent apoptosis in MCF7 but not in MCF7E6 cells, suggesting that p53 plays a vital role in apoptosis induced by chemotherapeutic agents. However, at a comparable expression level, p53 cannot cooperate with DNA damage agents to induce apoptosis in MCF7 cells (for detail, please see manuscript #1).

Tasks 4-5: The role of cyclin D1 in apoptosis. As we discussed in the fiscal year 2000 annual report, our preliminary data showed that transient overexpression of cyclin D1 in MCF7 cells can enhance DNA damage-induced p53-dependent apoptosis. We also found that transient co-expression of p53 and cyclin D1 in MCF7 cells can induce a strong apoptotic response. Since similar results have been published by other group (7), we decide to spend more effort on tasks 6-10 and we have made many interesting observations and contributions.

Tasks 6-7: Determination of minimum region in p53 necessary for inducing apoptosis. We have generated a number of p53 mutants and found that the both N- and C-terminal regions are dispensable for apoptosis. In addition, we precisely mapped the domains necessary for inducing apoptosis, cell cycle arrest, or both (Foe detail, please see manuscripts #2, 3, and 4). As an extension of these tasks, we also analyze the activities and functional domains of the p53 family members p63 and p73 (for detail, please see manuscripts #11 and 12).

Task 8-10: Identification of p53-interacting proteins and potential mediators for p53-dependent apoptosis. We showed that p73 functionally interacts with p53 in cells and activation of the p53 pathway is necessary for the cooperative induction of apoptosis between p73 and DNA damage in MCF7 cells (For detail, please see manuscript #1). As an extension of these tasks, we have identified several p53 target genes, including p73, MCG10, DKK1, TAP1, and AQP3. These p53 target genes are capable of mediating p53 tumor suppression in certain circumstances (for detail, please see manuscripts #5, 6, 7, 8, and 10).

Key Research Accomplishments for the award

1. A small but potent p53(ΔAD1ΔBD) molecule was generated, which can induce a strong apoptotic response in MCF7 cells.

2. We precisely mapped the domains in p53 necessary for inducing cell cycle arrest and apoptosis.
3. We found that chemotherapeutic agents cooperate with p73 to induce apoptosis in MCF7 breast cancer cells in a p53-dependent manner.
4. We found that p53 induces the transporter associated with antigen processing 1 gene and enhances the transport of MHC I class I peptides, suggesting that p53 plays a role in immunosurveillance.
5. We found that p53 induces Dickkopf-1, an inhibitor of the Wnt signaling pathway, suggesting that Dickkopf-1 may mediate p53 tumor suppression by antagonizing the Wnt signaling pathway.
6. We found that p53 induces MCG10, a KH domain RNA binding protein, which is capable of inducing apoptosis and cell cycle arrest in G2-M.
7. We found that the p53 family member p73 is regulated by DNA damage, p53, and p73.
8. We found that AQP3, a glycerol and water transporter, is upregulated by p73 of the p53 family.
9. We characterized the functional domains and activities of the p53 family members p63 and p73 and found that p63 and p73 have similar activities to p53 but also have some important differences.

Reportable Outcomes (Bibliography) for the award

Manuscripts

1. Zhu, J., S. Nozell, J. Wang, J. Jiang, W. Zhou, and **X. Chen.** 2001. p73 cooperates with DNA damage agents to induce apoptosis in MCF7 cells in a p53-dependent manner. ***Oncogene In Press.***
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Abstracts and presentations

13. A presentation at Cancer genetics and tumor suppressor genes meeting at Cold Spring Harbor, New York. August 19-23, 1998. Abstract #51.
14. A presentation at the 90th American Association for Cancer research annual meeting, Philadelphia, PA. April 10-14, 1999. Abstract #677.
15. A presentation at the 91st American Association for Cancer Research annual meeting, San Francisco, CA. April 1-5, 2000. Abstract #3958.
16. A presentation at the DOD Breast Cancer Research Program Meeting, Era of Hope, Atlanta, GA. June 8-11, 2000. Abstract # B-77.
17. A presentation at the 10th p53 workshop, Monterey, CA. April 5-8, 2000. Abstract #32.
18. A presentation at the 92nd American Association for Cancer Research (AACR) annual meeting, New Orleans, LA. March 24-28, 2001. Abstract #3958.

Funding applied for based on work supported by this award

NIH/NCI. RO1CA81237. Mechanism of p73-dependent tumor suppression. May 1999 - April 2004.

Pending: NIH/NCI. RO1CA95234. The functional domains and activities of the p53 family member p63.

Conclusions

We have precisely mapped the functional domains necessary for inducing apoptosis and cell cycle arrest (33-35). We also found that the apoptotic activity in p53 requires its transcriptional activity (35). Furthermore, we have generated p53(ΔAD1ΔBD), which lacks the MDM2 binding site and would not be subjected to the negative regulation by MDM2 (22). Thus, p53(ΔAD1ΔBD) represents a small but potent, apoptosis-inducing form of p53 for the MCF7 breast cancer cells (34). Recent clinical tries have shown that adenoviruses expressing p53 are effective in treating some advanced forms of human cancers (6, 27). We suggest that p53(ΔAD1ΔBD) is a good candidate to replace the larger, unwieldy wild-type p53 in cancer gene therapy.

We found that DNA damage agents cooperate with p73 to induce apoptosis in a p53-dependent manner. Since p73, unlike p53, is not frequently mutated in human cancer (4, 15, 17), it has been hoped that p73, or agents that can activate the p73 pathway, may be used as therapeutic agents for p53-defective tumors. Thus, the finding here should be taken into consideration if p73 is to be used as a potential therapeutic agent.

We found that like p53, p63 and p73 can induce apoptosis and cell cycle arrest. While some of the signaling pathways are shared among the entire p53 family, p63 and p73 each can induce unique target genes. These findings indicate that more studies are needed to address the unique activities of p63 and p73.

We have identified several novel target genes for the p53 family, including TAP1. The identification of TAP1 as a novel p53 and p73 target gene suggests that p53 and p73 function as tumor suppressors by regulating host immunosurveillance. Supporting this idea is the observation that deficiencies in p53 and p73 render mice susceptible to chronic infections, inflammation, and death due to unresolved infections. Therefore, this finding will lead us to further determine the role of immunosurveillance in p53 and p73 tumor suppression.

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Appendix 1

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ORIGINAL PAPERS

p73 cooperates with DNA damage agents to induce apoptosis in MCF7 cells in a p53-dependent manner

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p73, a member of the p53 family, can induce apoptosis in cancer cells. Since p53-mediated apoptosis can be augmented by various cancer chemotherapeutic agents, it has been hypothesized that the status of the endogenous p53 gene in cancer cells is a key determinant in the outcome of cancer therapy. To determine whether p73 can sensitize cancer cells to apoptosis by DNA damage agents, several MCF7 adenocarcinoma cell lines that inducibly express p73 or p53 under a tetracycline-regulated promoter were generated. We found that at relevant physiological levels, p73, but not p53, is capable of sensitizing MCF7 cells to apoptosis induced by chemotherapeutic agents. In addition, we found that p73 can cooperate with the DNA damaging agent camptothecin to activate the initiator caspase 2. Furthermore, we found that p73 can cooperate with DNA damaging agents or p53 to induce some p53 target genes and activate their promoters. In contrast, in MCF7E6 cells that ectopically express the human papillomavirus E6 oncogene and are functionally p53-null, the ability of p73 to sensitize cells to apoptosis is abrogated. Taken together, these results suggest that a functional interaction between p53 and p73 in MCF7 cells leads to enhanced induction of apoptosis. *Oncogene* (2001) **00**, 000–000.

Keywords: p53; p73; DNA damage; apoptosis

Introduction

p73 is highly similar to p53, especially in the central sequence-specific DNA binding domain, the amino terminal activation domain, and the carboxyl terminal tetramerization domain (Chen, 1999; Kaelin, 1999b; Kaghad *et al.*, 1997; Yang, 2000). The p73 gene is expressed as at least six alternatively spliced forms, that is, p73α, p73β, p73γ, p73δ, p73ε, and p73ζ (De Laurenzi *et al.*, 1998; Kaghad *et al.*, 1997; Laurenzi *et al.*, 1999; Zaika *et al.*, 1999). Like p53, p73 can induce cell cycle arrest and apoptosis when over-expressed in cells (Jost *et al.*, 1997; Kaghad *et al.*, 1997; Zhu *et al.*, 1998a). As a sequence-specific transcription factor, p73 can recognize several p53 response elements both *in vitro* and *in vivo* (Jost *et al.*, 1997; Kaghad *et al.*, 1997; Zhu *et al.*, 1998a). Loss of p73 transcriptional activity abrogates its ability to induce cell cycle arrest and apoptosis (Jost *et al.*, 1997; Kaghad *et al.*, 1997; Zhu *et al.*, 1998a). Despite these similarities to p53, p73 differentially regulates some p53 target genes (Di Como *et al.*, 1999; Marin *et al.*, 1998; Yu *et al.*, 1999; Zhu *et al.*, 1998a). For example, p21, a cyclin-dependent kinase inhibitor that is primarily responsible for p53-dependent G_1 arrest (Agarwal *et al.*, 1998; Ko and Prives, 1996), is regulated by p73, but the induction by p73 is several fold lower than by p53. Interestingly, 14-3-3σ, which may mediate p53-dependent G_2-M arrest (Hermeking *et al.*, 1997), is induced several fold higher by p73 than by p53. These results suggest that the signaling pathways for p53 and p73 in inducing cell cycle arrest and apoptosis are similar but also have important differences.

The ability of p53 to induce cell cycle arrest and apoptosis can be activated by both intracellular and environmental stresses, e.g., DNA damage agents and hypoxia, in cells that carry an endogenous wild-type p53 gene (Giaccia and Kastan, 1998; Ko and Prives, 1996). Some of the agents that can activate the p53 pathway are currently used for cancer chemotherapy (O'Connor *et al.*, 1997; Weinstein *et al.*, 1997). In fact, the efficacy of cancer chemotherapy by some therapeutic agents is dependent upon their ability to activate the p53 pathway (O'Connor *et al.*, 1997; Weinstein *et al.*, 1997). Therefore, tumor cells that lack a functional p53 are more resistant to chemotherapeutic agents than cells that contain a functional p53, but can be sensitized when reconstituted with wild-type p53 (Chen *et al.*, 1996; Gallardo *et al.*, 1996; Mukhopadhyay and Roth, 1997; Wang *et al.*, 1998).

Since p53, but not p73, is frequently mutated in human cancers (Chen, 1999; Ko and Prives, 1996; Levine, 1997), it is anticipated that p73 may be used to sensitize p53 deficient tumor cells to cancer therapeutic agents. In this study, we found that p73 cooperates with DNA damage to induce apoptosis in MCF7 breast adenocarcinoma cells that carry an endogenous wild-type p53 gene. In addition, we found that activation of the p53 pathway is necessary for the cooperative induction of apoptosis and cellular target

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genes by p73 and DNA damage. This result should be taken into consideration when p73 is used as a cancer therapeutic agent.

Results

p53 can sensitize many types of cancer cells, for example, hematopoietic and lymphoid cells, to apoptosis by DNA damaging agents (Lowe *et al.*, 1994; O'Connor *et al.*, 1997). However, MCF7 cells are less prone to the p53-dependent DNA damage-induced apoptosis. To examine whether p73 can sensitize MCF7 cells to apoptosis by DNA damaging agents, we generated several groups of cell lines that: (i) inducibly express p73α, p73β, or p73α292; (ii) stably express the human papillomavirus (HPV) E6 oncogene and inducibly express p73; and (iii) inducibly express p53. Since the HPV E6 oncogene facilitates the ubiquitin-mediated degradation of p53, the MCF7 cells that express E6 oncogene (MCF7E6) become p53-null-like. By tagging p73 and p53 with an HA epitope, the relative amount of the exogenous p73 and p53 proteins can be quantified by Western blot analysis with rabbit anti-HA polyclonal antibody. After normalized to the level of actin, we found that comparable levels of HA-p73 and HA-p53 were expressed in MCF7-HA-p73α-2, MCF7E6-HA-p73α-45, and MCF7-HA-p53-7 cells when induced (Figure 1). We also used several commercial anti-p73 antibodies to detect the endogenous p73 protein in MCF7 cells. We were unable to detect it. This is probably due to the quality of the antibodies and/or the level of the p73 protein expressed in MCF7 cells.

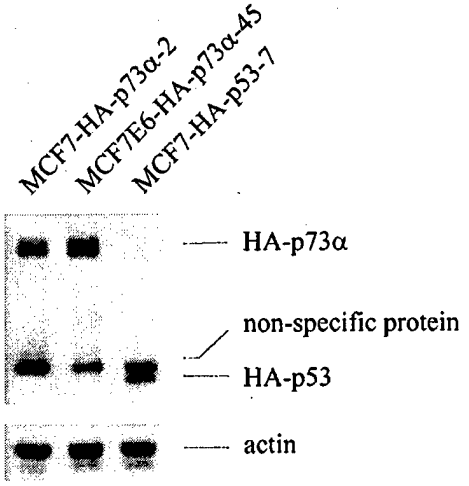

Figure 1 Comparable levels of the HA-tagged p73 and p53 are expressed in MCF7 cell lines that inducibly express p73 or p53, and in an MCF7E6 cell line that stably expresses HPV E6 oncogene and inducibly expresses p73. Cells were induced to express p73 or p53 for 24 h, and the levels of p73, p53, and actin proteins were determined by Western blot analysis. Polyclonal anti-HA antibody was used to detect the HA-tagged p73 and p53, and anti-actin polyclonal antibody was used to detect actin

To determine whether these MCF7 derivative cells still contain a functional p53 pathway and whether exogenous HA-p73 and HA-p53 are functonal, we analysed the expression levels of endogenous p53 and p21 following induction of exogenous HA-p73 or HA-p53, DNA damage, or both. We found that when treated with camptothecin, p53 was activated, leading to induction of p21 in MCF7 cells (Figure 2a,c, p53 and p21 panels, compare lanes 1 and 2). In contrast, p53 was not stabilized in MCF7E6 cells when treated with camptothecin, but p21 was slightly induced, presumably in a p53-independent manner (Figure 2b, p53 and p21 panels, compare lanes 1 and 2). When induced by withdrawal of tetracycline, HA-p73α was expressed in both MCF7 and MCF7E6 cells (Figure 2a,b, p73α panels, compare lane 1 with lane 4) and HA-p53 was expressed in MCF7 cells (Figure 2c, p53 panel, compare lane 1 with lane 4). Note that the HA-p53 migrated slowly compared to the endogenous p53 (Figure 2c, p53 panel, lane 4), which was slightly stabilized when exogenous HA-p53 was induced. Both HA-p73 and HA-p53 were capable of inducing p21 expression (Figure 2a–c, p21 panels, compare lane 1 with lane 4). When cells were treated with camptothecin and induced by withdrawal of tetracycline, both endogenous p53 and exogenous HA-p73α or HA-p53 were expressed in MCF7 cells (Figure 2a,c, p73α and p53 panels, lane 3). However, in MCF7E6 cells, only exogenous HA-p73α was expressed (Figure 2b, p73α and p53 panels, lane 3). In addition, we found that exogenous HA-p73α was slightly stabilized in both MCF7 and MCF7E6 cells when treated with camptothecin (Figure 2a,b, p73α panels, compare lanes 3 and 4). When the level of p21 was measured, we found that endogenous p53 and exogenous HA-p73α in MCF7 cells cooperated to induce p21 (Figure 2a, p21 panel, compare lanes 2, 3 and 4). p21 was also further induced in MCF7E6 cells when both treated with camptothecin and induced to express HA-p73α (Figure 2b, p21 panel, compare lanes 2, 3 and 4). This is probably due to an increased level of HA-p73α stabilized by DNA damage. However, the DNA damage induction of p21 in MCF7 cells was not substantially elevated by exogenous HA-p53 (Figure 2c, p21 panel, compare lane 3 with lanes 2 and 4).

We would like to emphasize here that the level of the inducible HA-p53 in MCF7 cells (Figure 2c) is lower than that of endogenous p53 stabilized by DNA damage (compare lanes 2 and 4) and can be further stabilized by DNA damage (compare lanes 3 and 4). Therefore, the effects of exogenous HA-p53 and possibly HA-p73 on cell proliferation and cell death are physiologically relevant.

Next, we determined whether exogenous HA-p73 and HA-p53 can sensitize MCF7 and MCF7E6 cells to apoptosis by DNA damage agents. We found that in the absence of either DNA damage or induction of HA-p73α, MCF7 and MCF7E6 cells grew normally (Figure 3a,e,i). When induced to express exogenous HA-p73α or HA-p53 for 3 days, both MCF7 and MCF7E6 cell proliferation were inhibited (Figure 3,

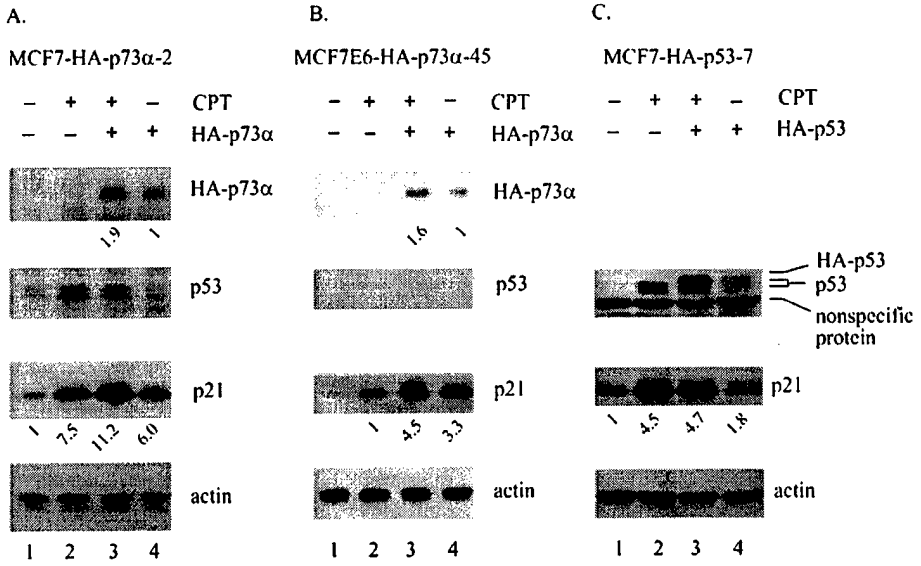

Figure 2 Characterization of MCF7 and MCF7E6 cell lines that inducibly express p73 and p53. MCF7 cells (a,c) or MCF7E6 cells (b) were uninduced (−) or induced (+) by withdrawal of tetracycline from culture media to express p73 or p53. Both uninduced and induced cells were mock-treated (−) or treated (+) with 300 nM camptothecin (CPT) for 24 h. The levels of p73, p53, p21, and actin proteins were determined by Western blot analysis with anti-p73 monoclonal antibody Ab-1, anti-p53 monoclonal antibody PAb240, anti-p21 monoclonal antibody Ab-1, and anti-actin polyclonal antibody. The fold of induction was shown below each blot

compare b with a; f with e; and j with i). When treated with 300 nM camptothecin to induce endogenous p53 but without inducing exogenous HA-p73α or HA-p53, MCF7 cells failed to multiply, enlarged and flattened (Figure 3, compare c and a; and k with i). In contrast, MCF7E6 continued to multiply albeit at a slightly slower rate than that of untreated cells (Figure 3, compare g and e). In addition, the treated MCF7E6 cells were not significantly enlarged and flattened (Figure 3, compare g with c and k). These results are consistent with previous observations that the p53 pathway is necessary for inhibiting cell proliferation following DNA damage (O'Connor *et al.*, 1997; Weinstein *et al.*, 1997). When cells were induced to express HA-p73α and treated with camptothecin to induce endogenous p53, MCF7 cells either became round or detached from the culture plate and shrank to form apoptotic bodies (Figure 3d). In contrast, DNA damage was incapable of cooperating with p73α in MCF7E6 cells to induce cell death, but cell proliferation was inhibited by HA-p73 (Figure 3h). In addition, we found that exogenous HA-p53 was incapable of cooperating with DNA damage to induce apoptosis in MCF7 cells (Figure 3). Similar results were obtained when these cells were treated with another therapeutic DNA damaging agent doxorubicin. We also tested MCF7 cells that inducibly express p73β or p73α mutant (p73α292). p73α292 is inert in transcriptional activity and defective in inducing cell cycle arrest and apoptosis (Jost *et al.*, 1997; Kaghad *et al.*, 1997; Zhu *et al.*, 1998a). When MCF7 cells were induced to express p73β or p73α292, DNA damage cooperated with p73β, but not mutant p73α292, to induce apoptosis. It should be noted that p73α, p73β, and p53 alone can induce a strong apoptotic response in several MCF7 cell lines when a higher level of p73 or p53 is expressed (Zhu *et al.*, 2000; data not shown). In addition, the p73-mediated apoptosis can be further enhanced by DNA damage (data not shown).

To quantify the extent of cell death to which exogenous HA-p73 or HA-p53 and DNA damage cooperate to induce in MCF7 cells, trypan blue dye exclusion assay was performed. We measured the percentage of dead cells three days after induction of HA-p73α or HA-p53, treated with camptothecin, or both in three separate experiments. In general, approximately 10% of control cells was stained positive (Figure 4a). This is probably due to trypsinization that was used to remove cells from culture plates. We found that the percentage of dead cells was slightly increased by treatment with camptothecin. While cell proliferation was inhibited by exogenous p73α and p53 in MCF7 cells (Figure 3b,f,j), no significant increase in cell death was detected (Figure 4a). However, p73, but not p53, cooperated to induce cell death for up to 40% of total cells in MCF7, but not MCF7E6, cells. The increase is nearly fourfold higher than that in untreated cells (Figure 4a).

To examine this further, we performed caspase activity assay. Since MCF7 cells harbor a dysfunctional caspase 3 gene (Nagata, 2000), we determined the activity of caspases 2 and 6. We found that when both treated with camptothecin and induced to express exogenous p73α, the activity of caspase 2 in MCF7

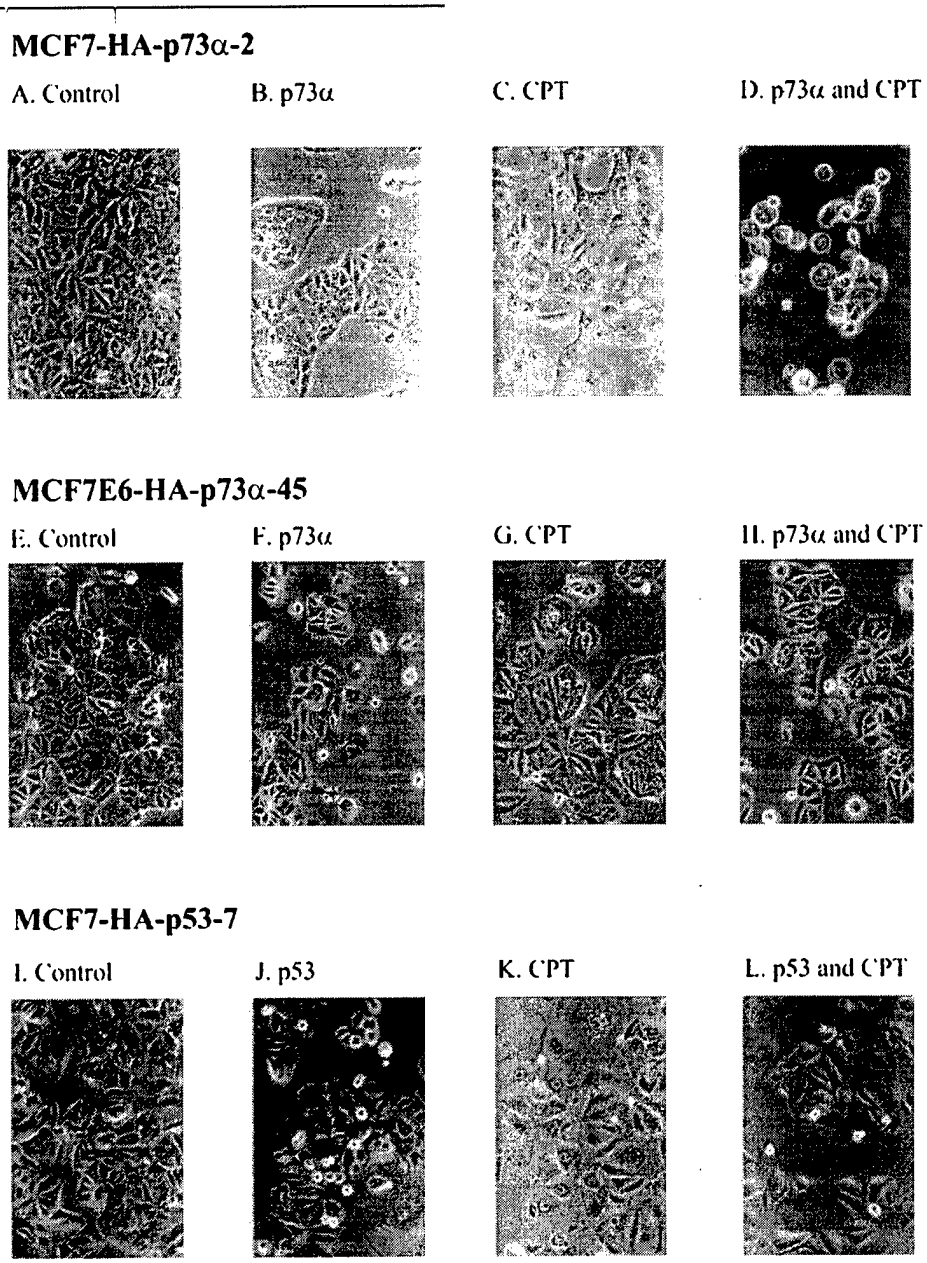

Figure 3 p73, but not p53, can sensitize MCF7, but not MCF7E6, cells to apoptosis by the DNA damaging agent camptothecin. MCF7 cells that inducibly express p73 were used in (**a–d**); MCF7E6 cells that stably express the HPV E6 oncogene and inducibly express p73 were used in (**e–h**); and MCF7 cells that inducibly express p53 were used in (**i–l**). These cells were mock-treated (**a,e,i**), induced to express p73 or p53 (**b,f,j**), treated with 300 nM camptothecin (**c,g,k**), and both treated with camptothecin and induced to express p73 or p53 (**d,h,l**). Magnification is 250 × for all panels

cells was increased to a substantially higher level than that in cells treated with camptothecin or induced to express p73α individually (Figure 4b). We also found that the activity of caspase 6 was similarly activated (data not shown). These results are consistent with the data obtained by the Trypan blue dye exclusion assay (Figure 4a).

Since the transcriptional activity of p73 is necessary for the enhanced induction of cell death in MCF7 cells treated with the DNA damaging agent camptothecin, it suggests that one or more cellular genes may be responsible for such cooperation. Indeed, p21 was induced cooperatively by exogenous p73 and DNA damage in MCF7 cells (Figure 2a). To further confirm this, Northern blot analysis was performed. We found that in MCF7 cells, *p21* was induced by DNA damage (Figure 5a,c, p21 panel), exogenous p73α (Figure 5a, p21 panel), or exogenous p53 (Figure 5c, p21 panel).

When MCF7 cells were treated with camptothecin and induced to express exogenous p73α or p53, *p21* was further induced (Figure 5a,c, p21 panel). In contrast, in MCF7E6 cells, *p21* was only slightly induced by DNA damage (~2.2-folds), presumably in a p53-independent manner (Figure 5b, p21 panel). Since the cell proliferation was not significantly repressed in MCF7E6 cells following DNA damage (Figure 3g), it appears that this level of p21 induced by DNA damage in MCF7E6 cells is not sufficient to arrest cells. However, in MCF7E6 cells, p21 was significantly induced by p73α (Figure 5b, compare lanes 1 and 4). In addition, *p21* was further induced when both treated with camptothecin and induced to express p73α (Figure 5b, p21 panel). This is probably due to an increased level of p73 stabilized by DNA damage (Figure 2b, p73 panel). Nevertheless, the fold of induction of p21 is still lower in MCF7E6 cells than that in MCF7 cells (Figure 5, p21 panel, compare b with a). These results are consistent with the data obtained by Western blot analysis (Figure 2), suggesting that up-regulation of p21 may be responsible for growth suppression by p73, p53 and DNA damage (Figure 3).

Next, we examined the expression of *BAX*, a pro-apoptotic p53 target gene. We found that BAX was not, or only slightly, induced by DNA damage, p73, and p53 individually (Figure 5, BAX panel). However, when treated with camptothecin and induced to express p73, a higher level of BAX was induced in MCF7 cells than in MCF7E6 cells (Figure 5a,b, BAX panel). In contrast, no further increase in BAX expression was detected in MCF7 cells by both DNA damage and exogenous p53 (Figure 5c, BAX panel). These results suggest that induction of BAX may be necessary for inducing apoptosis in MCF7 cells.

To further test that p53 and p73 cooperate to activate p53 target genes, we performed a luciferase reporter assay and analysed the regulation of p21 promoter by p53 and p73. We found that the activity of p21 promoter induced by both p53 and p73 was much higher than the additive by p53 and p73

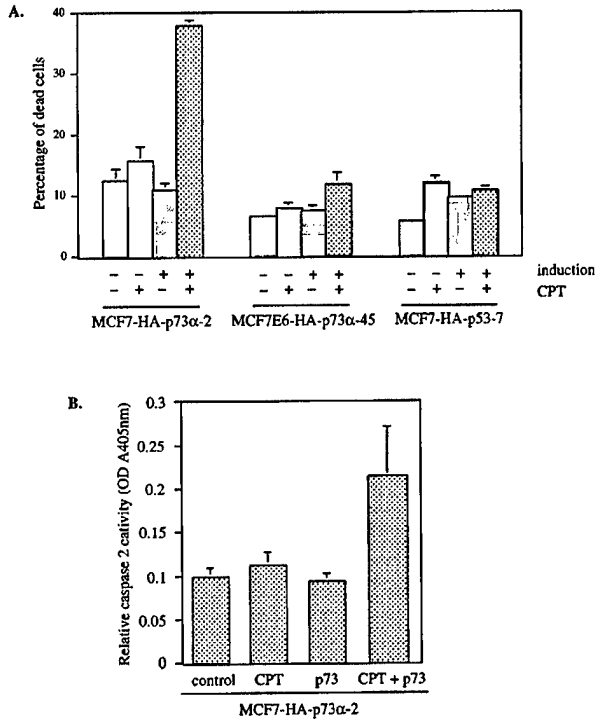

Figure 4 p73 and DNA damage cooperate to induce apoptosis in MCF7 cells. (**a**) MCF7 and MCF7E6 cells, that were uninduced (−) or induced (+) to express p73 or p53, were mock-treated (−) or treated (+) with 300 nM camptothecin (CPT) for 3 days. Following Trypan blue dye staining, both live and dead cells were counted and the percentage of dead cells was calculated. (**b**) Caspase 2 is activated in MCF7 cells when both treated with camptothecin and induced to express p73. The activity of caspase 2 was measured as described in Materials and methods

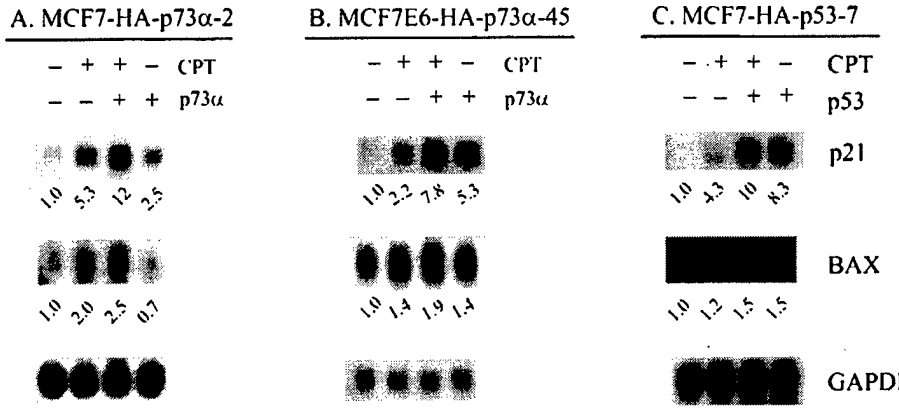

Figure 5 Regulation of cellular target genes by DNA damage, p73, and p53 in MCF7 and MCF7E6 cells. Total RNA was isolated from MCF7 and MCF7E6 cells that were either mock-treated (−) or treated (+) with 300 nM camptothecin (CPT) in the absence (−) or presence (+) of p73 or p53. Northern blots were then prepared using 10 μg total RNA and probed with cDNAs corresponding to the *p21*, *BAX*, and *GAPDH* genes, respectively. The fold of induction was shown below each blot

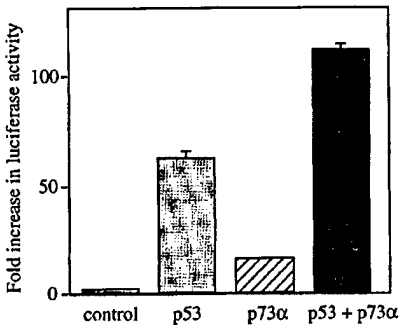

Figure 6 p73 and p53 cooperate to activate the promoter of the p21 gene. The p21-luc reporter vector was co-transfected with pCDNA3 control vector, a pcDNA3 vector that expresses p53 or p73, or a combination of two pcDNA3 vectors that express p53 and p73, respectively. Dual luciferase assay was performed according to the manufacturer's instruction (Promega). The fold increase in relative luciferase activity is a product of the luciferase activity induced by p53, p73, or both divided by that induced by pcDNA3

individually (Figure 6). Similarly, the promoter of the BAX gene was cooperatively activated by p53 and p73 (data not shown).

Discussion

Many studies have shown that tumor cells with an endogenous wild-type p53 gene are generally more sensitive to ionizing radiation and DNA damaging agents than tumor cells with a dysfunctional p53 gene (Chresta *et al.*, 1996; Lee and Bernstein, 1993; Lowe *et al.*, 1993, 1994; O'Connor *et al.*, 1997; Weinstein *et al.*, 1997). The underlying mechanism for such outcomes may simply be due to the fact that DNA damage can stabilize p53 and convert a 'latent' inactive form of p53 to an active one (Giaccia and Kastan, 1998; Prives and Hall, 1999), leading to apoptosis in tumor cells. In addition, overexpression of exogenous p53 can sensitize many cells that are p53-null, for example, H1299 cells, Saos-2 osteosarcoma cells, and SK-OV-3 ovarian cells, to apoptosis induced by DNA damaging agents (Chen *et al.*, 1996; Gallardo *et al.*, 1996; Wang *et al.*, 1998). Here we found that p73 cooperates with several DNA damaging agents to induce apoptosis in MCF7, but not in functionally p53-null MCF7E6, cells. Furthermore, p73-mediated apoptosis is inhibited in p53-null H1299 cells when treated with several DNA damaging agents (data not shown). Since p73, unlike p53, is not frequently mutated in human cancer (Chen, 1999; Ichimiya *et al.*, 1999; Kaelin, 1999a), it has been hoped that p73, or agents that can activate the p73 pathway, may be used as therapeutic agents for p53-defective tumors. Thus, the finding here should be taken into consideration if p73 is to be used as a potential therapeutic agent.

Several reports have shown that p73 is stabilized in cells by DNA damage in a c-Abl-dependent manner (Agami *et al.*, 1999; Gong *et al.*, 1999; Yuan *et al.*, 1999). In MCF7 cells, the c-Abl pathway is functional (Agami *et al.*, 1999), In addition, we have found that p73 can be stabilized by DNA damage in MCF7E6 cells (Figure 2), which express the HPV E6 protein. Thus, HPV E6 does not have adverse effect on the ability of the c-Abl pathway to stabilize p73. These results indicate that the p53, but not the c-Abl, pathway is responsible for the differential effect of DNA damage on p73-mediated apoptosis between MCF7 and MCF7E6 cells.

Recently, several studies have shown that p73 differentially regulates some of the p53 target genes (Di Como *et al.*, 1999; Marin *et al.*, 1998; Yu *et al.*, 1999; Zhu *et al.*, 1998a). Here we found that p73 cooperates with DNA damage (i.e., induction of p53) in MCF7 cells to induce endogenous p21 and BAX, and p53 and p73 cooperate to activate the p21 promoter. Several reports have shown that p73 can bind to the p53 response element in the promoter of several target genes (Di Como *et al.*, 1999; Marin *et al.*, 1998) and a protein complex containing both p53 and p73 has been detected (Di Como *et al.*, 1999; Marin *et al.*, 2000). Since the promoters for most p53 target genes, for example, the p21 promoter (Resnick-Silverman *et al.*, 1998), contain two p53 response elements, it is possible that p53 and p73 may separately bind to one of the two response elements. Interaction of p53 and p73 on the promoters would then cooperate to induce their target genes. Such functional interactions have been observed between Sp1 and Smad family proteins on the p21 promoter (Moustakas and Kardassis, 1998; Xiao *et al.*, 1999). Therefore, future studies using chromatin immunoprecipitation assay are needed to determine whether p53 and p73 form a hetero-complex on, or bind separately to, the promoter of cellular target genes *in vivo*.

Previous studies have shown that when MCF7 cells are subjected to ionizing radiation or treatment with DNA damaging agents, the endogenous p53 protein is stabilized and activated (Fan *et al.*, 1995; Gupta *et al.*, 1997; O'Connor *et al.*, 1997), leading to induction of p21 and cell cycle arrest. However, unlike hematopoietic and lymphoid cells that are readily undergoing an apoptotic response to DNA damage, MCF7 cells are less prone to the p53-dependent DNA damage-induced apoptosis. In addition, disruption of p53 function can sensitize MCF7 cells to the G_2-checkpoint abrogators, taxol and pentoxifylline (Fan *et al.*, 1995). Similarly, we found that the DNA damaging agent camptothecin stabilized and activated p53 in MCF7 cells but no significant apoptosis was detected by treatment with camptothecin (Figure 3) or doxorubicin in this study. Furthermore, exogenous p53 at a physiological relevant level, alone or in combination with DNA damaging agents, is unable to elicit an apoptotic response. However, we found that p73 cooperates with DNA damage to induce a strong apoptotic response in MCF7 but not in MCF7E6 cells, suggesting that p53 is necessary for cooperating with other apoptotic stimuli, such as p73, to induce apoptosis in MCF7 cells.

Materials and methods

Plasmids

cDNAs for p73α (Jost *et al.*, 1997) and p53 were cloned separately into a tetracycline-regulated expression vector, 10-3, at its *Eco*RI and *Xba*I sites and the resulting plasmids were used to generate cell lines that inducibly express p73 or p53. Both p53 and p73 were tagged at their N-termini with an influenza hemagglutinin (HA) peptide. cDNA for the human papillomavirus E6 gene (Munger *et al.*, 1989) was cloned into pBabe expression vector at its *Eco*RI and *Bam*HI sites (Morgenstern and Land, 1990).

Cell lines and DNA damage

The MCF7 cell line, which expresses tet-VP16 for generation of tetracycline inducible cell lines, was purchased from ClonTech (Palo Alto, CA, USA). MCF7 cell lines that express inducible proteins of interest were generated as previously described (Zhu *et al.*, 2000). Individual clones were screened for inducible expression of p73 protein by Western blot analysis using monoclonal antibody 12CA5 (Boehringer Mannheim Biochemical, Germany). To induce double-strand DNA breaks, cells were treated with camptothecin and doxorubicin (Sigma, St. Louis, MO, USA).

Western blot analysis

Cells were collected from plates in phosphate-buffered saline (PBS), resuspended with 1 × sample buffer, and boiled for 5 min. Western blot analysis was performed as described (Chen *et al.*, 1995), with rabbit anti-HA polyclonal antibody (Sigma, St. Louis, MO, USA), anti-p53 monoclonal antibody Pab240, affinity-purified anti-actin polyclonal antibody (Sigma, St. Louis, MO, USA), anti-p73α polyclonal antibody (Ab4) (Oncogene Research Products, Cambridge, MA, USA), or anti-p21 monoclonal antibody (Ab-1) (Oncogene Research Products, Cambridge, MA, USA).

Trypan blue dye exclusion assay

Cells were seeded at 2×10^5 per 90-mm plate. After adhering to culture plates, cells were mock-treated or treated with camptothecin or doxorubicin in the presence or absence of tetracycline. Three days after treatment, both floating cells in the medium and live cells on the plate were collected and concentrated by centrifugation. After staining with Trypan blue dye (Sigma) for 15 min, both live (unstained) and dead (stained) cells were counted three times in a hemocytometer. The percentage of dead cells was calculated and used as an index for the degree of cell death.

Caspase activity assay

Cells were seeded at $3-5 \times 10^5$ per 90-mm plate in the presence or absence of tetracycline, which were also mock-treated or treated with 300 nM camptothecin for three days. Adherent cells were then rinsed with cold PBS and the caspase activity was assayed using the caspases 2 and 6 colorimetric protease assay reagents according to the manufacturer's instruction (Chemicon International, Inc.). The percentage of increase of relative caspase activity was a product of the activity in cells, which were treated with camptothecin, induced to express p73, or both, divided by that in control cells.

RNA isolation and Northern blot analysis

Total RNA was isolated using Trizol reagents (GIBCO-BRL). Northern blot analysis was performed as described (Zhu *et al.*, 1998b). The *p21*, *BAX*, and *GADPH* probes were prepared as previously described (Zhu *et al.*, 1998b).

Luciferase assay

The $p21^{WAF1}$ promoter was cloned upstream of a luciferase reporter gene (Chinery *et al.*, 1997). 1.0 μg of the resulting reporter vector was cotransfected into H1299 cells with 0.5 μg of pcDNA3 control vector or a vector expressing p53, p73α, or both. For an internal control, 25 ng of the Renilla luciferase vector pRL-CMV (Promega, Madison, WI, USA) were cotransfected with the above constructs. Dual luciferase assays were performed in triplicate according to the manufacturer's instructions (Promega, Madison, WI, USA).

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Appendix 2

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Identification of a Novel p53 Functional Domain That Is Necessary for Mediating Apoptosis*

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The ability of p53 to induce apoptosis requires its sequence-specific DNA binding activity; however, the transactivation-deficient p53(Gln^{22}-Ser^{23}) can still induce apoptosis. Previously, we have shown that the region between residues 23 and 97 in p53 is necessary for such activity. In an effort to more precisely map a domain necessary for apoptosis within the N terminus, we found that deletion of the N-terminal 23 amino acids compromises, but does not abolish, p53 induction of apoptosis. Surprisingly, p53(Δ1–42), which lacks the N-terminal 42 amino acids and the previously defined activation domain, retains the ability to induce apoptosis to an even higher level than wild-type p53. A more extensive deletion, which eliminates the N-terminal 63 amino acids, renders p53 completely inert in mediating apoptosis. In addition, we found that both p53(Δ1–42) and p53(Gln^{22}-Ser^{23}) can activate a subset of cellular p53 targets. Furthermore, we showed that residues 53 and 54 are critical for the apoptotic and transcriptional activities of both p53(Δ1–42) and p53(Gln^{22}-Ser^{23}). Taken together, these data suggest that within residues 43–63 lie an apoptotic domain as well as another transcriptional activation domain. We therefore postulate that the apoptotic activity in p53(Gln^{22}-Ser^{23}) and p53(Δ1–42) is still transcription-dependent.

The p53 tumor suppressor protein serves as a checkpoint in maintaining genome stability (1–3). Several different biological responses that could play a role in maintaining genome stability have been strongly correlated with wild-type p53 function (1, 3). Following stress conditions such as in the presence of damaged DNA or insufficient growth and survival factors, the cellular levels of p53 increase. This leads to one of at least three well understood cellular responses as follows: cell cycle arrest, differentiation, or apoptosis. Several factors have been shown to determine how a cell responds to the accumulation of p53, *e.g.* cell type and the presence of several cellular and viral proteins (4–8). In addition, the levels of p53 in a given cell can dictate the response of the cell such that lower levels of p53 result in cell cycle arrest (9) or differentiation (10), whereas higher levels result in apoptosis (9, 10).

The functional domains of p53 have been subjected to extensive analysis (1, 3, 4). A transcriptional activation domain has been shown to lie within N-terminal residues 1–42 (11, 12). Within this region there are a number of acidic and hydrophobic residues, characteristics of the acidic activator family of transcriptional factors (13). Indeed, a double point mutation of the two hydrophobic amino acids at residues 22 and 23 renders p53 transcriptionally inactive (14). These two residues presumably are required for the interaction of the activation domain with the TATA box binding protein and/or TATA box binding protein-associated factors (15–18).

It is well established that as a transcriptional activator, p53 up-regulates p21, a cyclin-dependent kinase inhibitor (19–21), which leads to p53-dependent G_1 arrest. However, it is not certain what function(s) of p53 is required for apoptosis. The transactivation function of p53 was shown to be required in some experimental protocols (22–24). There are several candidate genes that play roles in apoptosis that can be activated in response to p53 induction, such as *BAX* (25), *IGFBP3* (26), *PAG608* (27), *KILLER/DR5* (28), and several redox-related *PIGs* genes (29). Several other studies, including our own observations, have provided evidence that p53 might have a transcription-independent function in apoptosis (9, 30–32). Recently, the proline-rich region between residues 60 and 90, which comprises five "PXXP" motifs (where P represents proline and *X* any amino acid), was found to be necessary for efficient growth suppression (33) and apoptosis (34) and to serve as a docking site for transactivation-independent growth arrest induced by GAS1 (35).

Previously, we showed that the region between residues 23 and 97 is necessary for apoptosis (9). To more precisely map such a domain in the N terminus necessary for apoptosis, we have made several new mutants. Analyses of these mutants lead to identification of a novel domain between residues 43 and 63 that can mediate apoptosis and activate cellular p53 targets. We also found that a double point mutation at residues 53 and 54 completely abolishes both the transcriptional and apoptotic activities mediated by this novel domain. Thus, we hypothesize that a transcriptional activity located in this novel domain regulates a subset of cellular p53 targets that are responsible for apoptosis.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis—Mutant p53 cDNAs were generated by polymerase chain reaction using the full-length wild-type p53 cDNA as a template. To generate p53(Δ1–23), the pair of primers used were as follows: forward primer N24, GAT CGA ATT CAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC TAA ACT ACT TCC TGA A; and reverse primer C393, GAT CGA ATT CTC AGT CTG AGT CAG GCC CTT. To generate p53(Δ1–42), the pair of primers used were as follows: forward primer N43, GAT CGA ATT CAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC TTT GAT GCT GTC CCC G; and reverse primer C393. To generate p53(Δ1–63), the pair of primers used were: forward primer N64, GAT CGA ATT CAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC TCC CAG AAT GCC AGA GGC T; and reverse primer C393. To generate p53(Gln^{22}-Ser^{23}/Gln^{53}-Ser^{54}), cDNA fragments encoding amino acids 1–59 and 60–393 were amplified in-

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dependently and ligated together through an internal *Ava*II site. The p53(Gln^{22}-Ser^{23}) cDNA was used as a template (14). The pair of primers for the cDNA fragment encoding amino acids 1–59 were as follows: forward primer N1, GAT CGA ATT CAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC TGA GGA GCC GCA GTC AGA TCC; and reverse primer C59, TTC ATC TGG ACC TGG GTC TTC AGT GCT CTG TTG TTC AAT ATC. The pair of primers for the cDNA fragment encoding amino acids 60–393 were as follows: forward primer N60, ACT GAA GAC CCA GGT CCA; and reverse primer C393. To generate p53(Δ1–42/Gln^{53}-Ser^{54}), the p53(Gln^{22}-Ser^{23}/Gln^{53}-Ser^{54}) cDNA was amplified by forward primer N43 and reverse primer C393. Mutations were confirmed by DNA sequencing.

The above mutant p53 cDNAs were cloned separately into a tetracycline-regulated expression vector, 10-3, at its *Eco*RI site, and the resulting plasmids were used to generate cell lines that inducibly express p53.

Cell Lines, Transfection, and Selection Procedures—The H1299 cell line was purchased from the American Type Culture Collection and grown with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C with 5% CO_2. Transfections were performed using the calcium chloride method as described (36). Cell lines expressing inducible proteins of interest were generated as described previously (9). Individual clones were screened for inducible expression of the p53 protein by Western blot analysis using monoclonal antibodies against p53. The H1299 cell lines that inducibly express either wild-type p53 or p53(Δ364–393) are p53-3 and p53(Δ364–393)-1, respectively, as described previously (9). The H1299 cell line that inducibly expresses p53(Δ62–91) is p53(Δ62–91)-5.[1]

Western Blot Analysis—Cells were collected from plates in phosphate-buffered saline, resuspended with 1× sample buffer, and boiled for 5 min. Western blot analysis was performed as described previously (37). Monoclonal antibodies used to detect p53 were Pab240 and Pab421 (37). The affinity purified monoclonal antibody against p21 (Ab-1) was purchased from Oncogene Science (Uniondale, NY). Affinity purified anti-actin polyclonal antibodies were purchased from Sigma.

Growth Rate Analysis—To determine the rate of cell growth, cells were seeded at 5–10 × 10^4 cells per 60-mm plate, with or without tetracycline (2 μg per ml). The medium was replaced every 72 h. At times indicated, two plates were rinsed with phosphate-buffered saline twice to remove dead cells and debris. Live cells on the plates were trypsinized and collected separately. Cells from each plate were counted three times by Coulter cell counter. The average number of cells from at least two plates were used for growth rate determination.

FACS[2] *Analysis*—Cells were seeded at 2.0 × 10^5 per 90-mm plate with or without tetracycline. Three days after plating, both floating dead cells in the medium and live cells on the plate were collected and fixed with 2 ml of 70% ethanol for at least 30 min. For FACS analysis, the fixed cells were centrifuged and resuspended in 1 ml of phosphate-buffered saline solution containing 50 μg/ml each of RNase A (Sigma) and propidium iodide (Sigma). The stained cells were analyzed in a fluorescence-activated cell sorter (FACSCaliber, Becton Dickinson) within 4 h. The percentage of cells in sub-G_1, G_0-G_1, S, and G_2-M phases was determined using the ModFit program. The percentage of cells in sub-G_1 phase was used as an index for the degree of apoptosis.

Cell Viability Assay by Trypan Blue Exclusion—Cells were seeded at 2 × 10^5 per 90-mm plate with or without tetracycline. Three days after plating, both floating cells in the medium and live cells on the plate were collected and concentrated by centrifugation. After stained with trypan blue (Sigma) for 15 min, both live (unstained) and dead (stained) cells were counted two times in a hemocytometer. The percentage of dead cells from control plates was subtracted from the percentage of dead cells from experimental plates, and the resulting value was used as an index for the degree of apoptosis.

RNA Isolation and Northern Blot Analysis—Total RNA was isolated using Trizol reagent (Life Technologies, Inc.). Northern blot analysis was performed as described (37). The *p21* probe was made from an 1.0-kilobase pair *Eco*RI-*Eco*RI fragment (19); the *MDM-2* probe was made from a 2.1-kilobase pair *Not*I-*Sma*I fragment (38); the *BAX* probe was made from a 290-base pair *Pst*I-*Bgl*II fragment (39); the *GADD45* probe was from a 400-base pair *Eco*RI-*Bam*HI fragment (40); the *GAPDH* probe was made from an 1.25-kilobase pair *Pst*I-*Pst*I cDNA fragment (41); and the *MCG14* cDNA probe was a 200-base pair polymerase chain reaction fragment identified by CLONTECH PCR-Select cDNA subtraction.[2]

[1] J. Zhu, W. Zhou, J. Jiang, and X. Chen, manuscript in preparation.
[2] The abbreviation used is: FACS, fluorescence-activated cell sorter.

RESULTS

A Novel Domain within Residues 43–63 Is Necessary for Mediating Apoptosis—Previously, we showed that p53(Δ1–22), which lacks the N-terminal 22 amino acids, can still induce apoptosis as well as cell cycle arrest (9). Since both residues 22 and 23 are critical for p53 transcriptional activity (14), we decided to determine whether p53(Δ1–23), which deletes the N-terminal 23 amino acids, would also be able to induce apoptosis and activate cellular p53 targets.

We have previously established a cell line that expresses high levels of wild-type p53 called p53-3 (9). This line was established using a tetracycline-regulated expression system as described previously (42). By using similar techniques, we established nine stable cell lines that express p53(Δ1–23). Three representative cell lines, p53(Δ1–23)-9, -10, and -23, are shown in Fig. 1*A*. Western blot analysis showed that these cell lines express p53(Δ1–23) at levels comparable to wild-type p53 in p53-3 cells (Fig. 1*A*). To characterize p53(Δ1–23), we looked at its transcriptional and apoptotic activities and the growth rate of the cell line p53(Δ1–23)-9. The transcriptional activity was determined by monitoring the expression of the endogenous gene, p21, a well defined transcriptional target of p53 (19). We found that p53(Δ1–23) is still capable of activating p21, albeit to a much less degree than wild-type p53 (Fig. 1*A*). Next, the growth rates of p53(Δ1–23)-9 cells under both uninduced and induced conditions were determined, and these cells failed to multiply following p53 expression (Fig. 1*B*). To exclude potential effects of the regulator tetracycline and/or the tet-vp16 transactivator (42) on cell growth, we analyzed the growth rate of the cell line H24-1, which was similarly established but did not express any protein. The results showed that the growth rates of H24-1 cells under both the uninduced (+tet) and induced (−tet) conditions were nearly identical (Fig. 1*C*), indicating that both tetracycline and tet-vp16 transactivator have no effect on cell growth. It is well established that the percentage of cells containing a sub-G_1 DNA content reflects the extent to which cells are undergoing apoptosis (9, 23, 31). Since p53 can induce apoptosis in H1299 cells (9, 31), FACS analysis was used to observe the extent of apoptosis by determining the distribution of cells in each phase of the cell cycle. The results showed that 18% of cells expressing p53(Δ1–23) had a sub-G_1 DNA content 3 days after induction of this mutant, compared with less than 5% of the same cells expressing no p53 (Fig. 1, *D* and *E*; Table I). Trypan blue exclusion assay showed that 15% of cells were dead, which is consistent with FACS analysis. In contrast, about 45 and 30% of cells had a sub-G_1 DNA content at day 3 following expression of either wild-type p53 or transactivation-deficient p53(Gln^{22}-Ser^{23}), respectively (Table I). The FACS results also showed that the number of cells in S phase was decreased from 38 to 22.3% following induction of p53(Δ1–23), and these cells primarily arrested in G_1 (Fig. 1, *D* and *E*). Similar results were obtained using another high p53(Δ1–23) producer, p53(Δ1–23)-10.

Since p53(Δ1–23) is still capable of inducing apoptosis and p53 activation domain lies within residues 1–42 (11, 12), we determined whether the other half (residues 24–42) of the previously defined activation domain is required for apoptosis. To this end, we established 16 individual stable cell lines that inducibly express p53(Δ1–42) that lacks the N-terminal 42 amino acids. Three representative cell lines, p53(Δ1–42)-2, -5, and -11, were shown in Fig. 2*A*. Consistent with previous results that p53(Gln^{22}-Ser^{23}) cannot activate p21 (9, 22, 33, 43), p53(Δ1–42) only minimally activated p21 as compared with wild-type p53 (Fig. 2*A*). We then determined the growth rate of a high producer, p53(Δ1–42)-2. Surprisingly, we found that a majority of cells died within 3 days following induction of

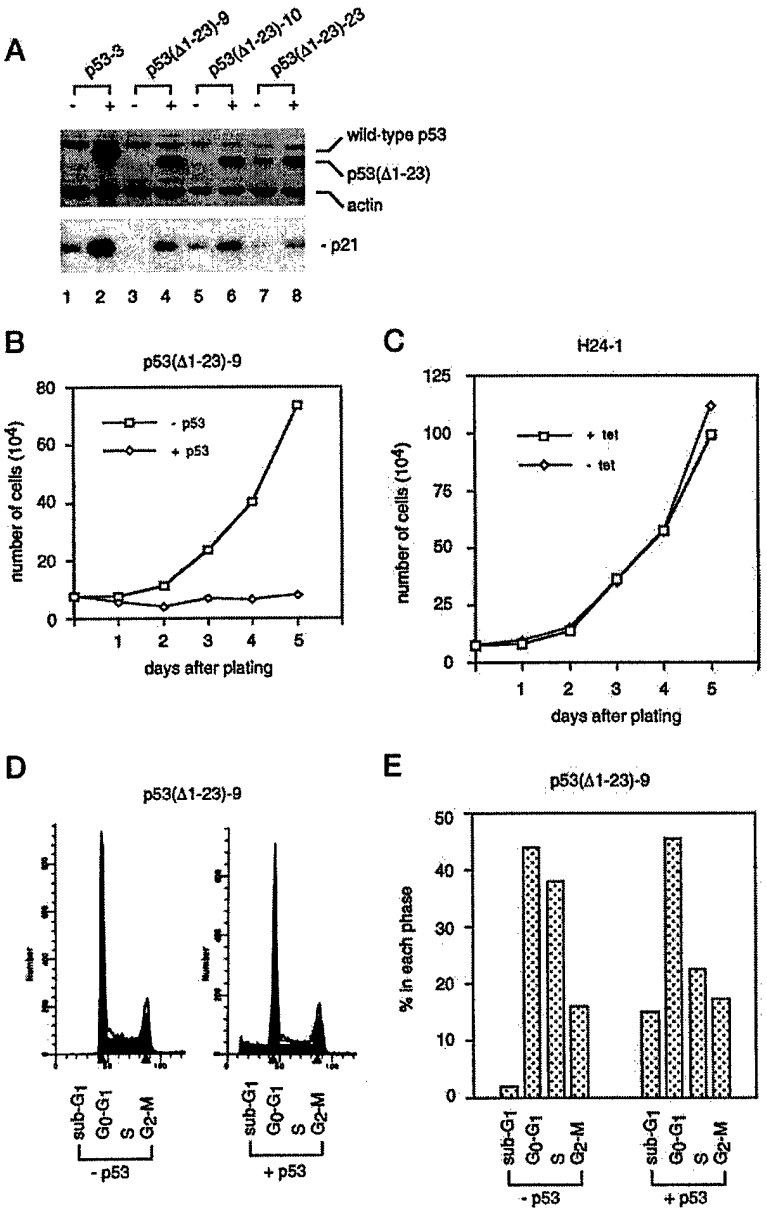

FIG. 1. **The N-terminal 23 amino acids are dispensable for apoptosis.** *A*, levels of p53, p21, and actin in p53-3, and p53(Δ1–23)-9, -10, and -23 cell lines were assayed by Western blot analysis. Cell extracts were prepared from uninduced cells (−) or cells induced to express (+) wild-type p53 or p53(Δ1–23). The upper portion of the blot was probed with a mixture of p53 monoclonal antibodies Pab421 and Pab240 and actin polyclonal antibody. Mutant p53(Δ1–23) migrates faster than wild-type p53 because it is missing 23 amino acids. The lower portion of the blot was probed with p21 monoclonal antibody. *B*, growth rates of p53(Δ1–23)-9 cells in the presence (◇) or absence (□) of p53 were measured as described under "Experimental Procedures." *C*, growth rates of H24-- cells in the presence (□) or absence (△) of tetracycline. *D*, DNA contents were quantitated by propidium iodide staining of fixed cells at day 3 following withdrawal of tetracycline as described under "Experimental Procedures." *E*, The percentages of p53(Δ1–23)-9 cells in sub-G_1, G_0-G_1, S, and G_2-M phases in the presence or absence of p53 for 3 days were quantitated using ModFit program as described under "Experimental Procedures."

TABLE I
Characteristics of various mutant p53 proteins

	Arrest[a]	Apoptosis[b]
Wild-type p53	+++	45
p53(Gln^{22}-Ser^{23})	−	30
p53(Gln^{22}-Ser^{23}/Gln^{53}-Ser^{54})	−	
p53(Δ1–22)	+++	>60
p53(Δ1–23)	++	15 –18
p53(Δ1–42)	+/−	50 –68
p53(Δ1–42/Gln^{53}-Ser^{54})	−	
p53(Δ1–63)	−	

[a] Arrest was assayed by the relative growth rate of cells and the number of cells in S phase.

[b] Apoptosis was assayed at day 3 by the percentage of cells staining with trypan blue and by determination of the sub-G_1 phase cells using the Modfit program.

p53(Δ1–42) (Fig. 2*B*). In addition, both trypan blue exclusion assay and FACS analysis showed that approximately 50–68% of cells underwent apoptosis (Fig. 2*C*; Table I). Similar results were obtained from several other cell lines. These results suggest that the entire previously defined activation domain within the N-terminal 42 amino acids is dispensable for apoptosis. In fact, deletion of this region enhanced the ability of p53 to induce apoptosis (Table I).

To delineate further the domain in the N terminus required for apoptosis, we generated seven inducible cell lines expressing p53(Δ1–63) which lacks the N-terminal 63 amino acids but contains an intact proline-rich region. Three representative cell lines, p53(Δ1–63)-14, -22, and -27, were shown in Fig. 3*A*, and the activity of p53(Δ1–63) was analyzed as above. The results showed that p53(Δ1–63) was unable to activate p21 expression (Fig. 3*A*), and p53(Δ1–63)-14 cells, a high p53 producer, continued to multiply when p53(Δ1–63) was induced (Fig. 3*B*). Furthermore, both FACS analysis and trypan blue exclusion assay showed that neither apoptosis nor cell cycle arrest was observed in cells expressing p53(Δ1–63) (Fig. 3*C* and Table I).

Within Residues 43–63 Lies Another Activation Domain That Overlaps with the Domain Necessary for Mediating Apoptosis—The ability of transactivation-deficient p53(Gln^{22}-Ser^{23}) to induce apoptosis leads to the hypothesis that p53 has transcription-independent apoptotic activity (9, 31, 33). Since p53(Δ1–42) lacks the previously defined activation domain and only

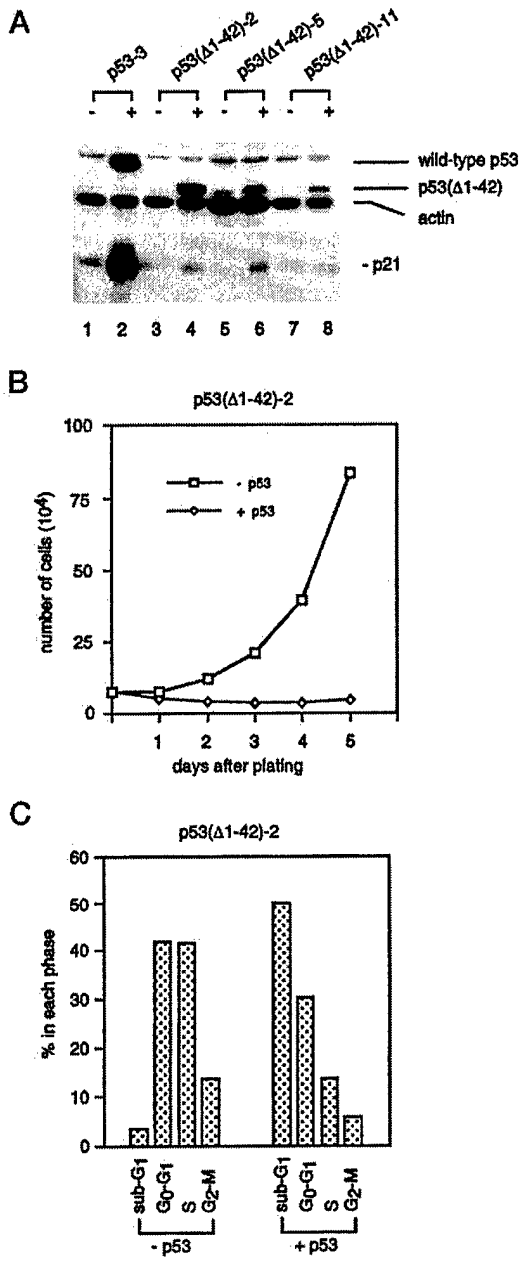

FIG. 2. **p53(Δ1–42), which lacks the previously defined activation domain, can mediate apoptosis.** *A*, levels of p53, p21, and actin in p53-3, and p53(Δ1–42)-2, -5, and -11 cell lines were assayed by Western blot analysis. *B*, growth rates of p53(Δ1–42)-2 cells in the presence (◇) or absence (□) of p53. *C*, the percentages of p53(Δ1–42)-2 cells in sub-G_1, G_0-G_1, S, and G_2-M phases in the presence or absence of p53 for 3 days. The experiments were performed in an identical manner to those in Fig. 1.

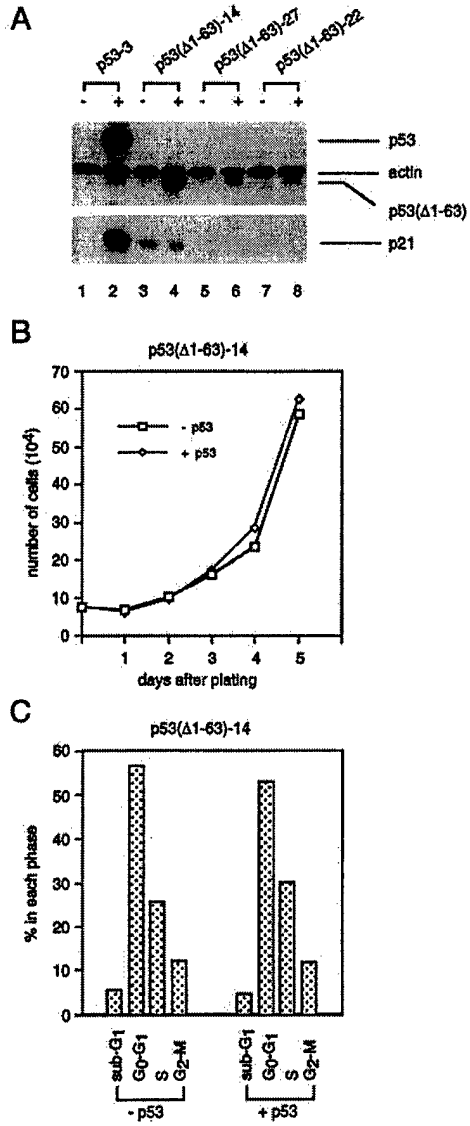

FIG. 3. **p53(Δ1–63), which lacks the N-terminal 63 amino acids but contains intact PXXP motifs, failed to induce apoptosis.** *A*, levels of p53, p21, and actin in p53-3, and p53(Δ1–63)-14, -22, and -27 cell lines were assayed by Western blot analysis. *B*, growth rates of p53(Δ1–63)-14 cells in the presence (◇) or absence (□) of p53. *C*, the percentages of p53(Δ1–63)-14 cells in sub-G_1, G_0-G_1, S, and G_2-M phases in the presence or absence of p53 for 3 days. The experiments were performed in an identical manner to those in Fig. 1.

minimally activates p21 as determined by Western blot analysis (Fig. 2*A*), it appears that it can induce apoptosis in a transcription-independent manner. To ascertain whether p53(Δ1–42) contains a transcriptional activity, the expression patterns of four well defined cellular p53 targets, *p21*, *MDM2*, *GADD45*, and *BAX*, were analyzed in cells expressing p53(Δ1–42) by Northern blot analysis (Fig. 4*A*). The expression levels of these genes in cells with or without p53 were quantitated by PhosphorImage scanner, and the fold increase of their relative mRNAs was calculated after normalization to glyceraldehyde-3-phosphate dehydrogenase mRNA levels (Table II). The results showed clearly that p53(Δ1–42) significantly activated *MDM2* (8-fold), *GADD45* (7.03-fold), and *BAX* (3.9-fold) but only minimally activated *p21* (1.83-fold). As expected, wild-type p53 but not mutant p53(Gln^{22}-Ser^{23}) activated these cellular p53 targets (Fig. 4*A*; Table II). As a control, p53(Δ64–91), which lacks all of the five PXXP motifs, was examined. The proline-rich domain in p53 is dispensable for transactivation (33, 34). As expected, p53(Δ64–91) activated these p53 targets (Fig. 4*A* and Table II). Since p53(Δ1–63) failed to activate any of these p53-regulated genes (data not shown), the results suggest that another activation domain lies within residues 43–63. For clarity, we designate the originally defined activation domain located within residues 1-42 as activation domain I and this novel domain as activation domain II.

The above observations raise the following question: why does p53(Gln^{22}-Ser^{23}) fail to activate these well-defined p53 transcriptional targets (Fig. 4*A*; Table II) despite the fact that it still contains an intact activation domain II? One of the

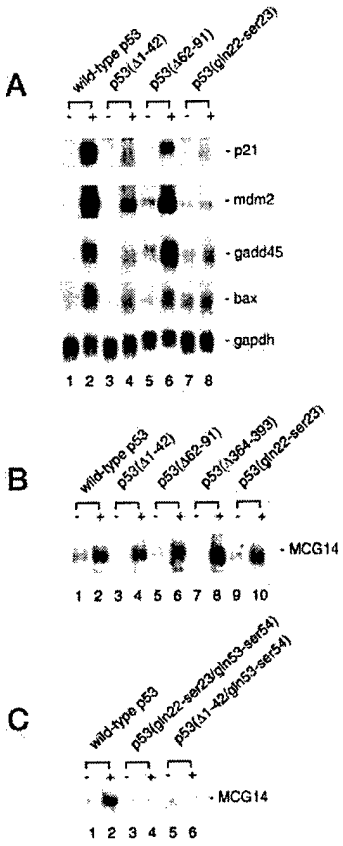

FIG. 4. **Within residues 43–63 lies another activation domain.** *A*, Northern blots were prepared using 10 μg of total RNA isolated from uninduced cells (−) or cells induced to express (+) wild-type p53, p53(Δ1–42), p53(Δ62–91), or p53(Gln^{22}-Ser^{23}). The blots were probed with *p21, MDM2, GADD45, BAX,* and *GAPDH* cDNAs, respectively. *B*, a Northern blot was prepared using 10 μg of total RNA isolated from uninduced cells (−) or cells induced to express (+) wild-type p53, p53(Δ1–42), p53(Δ62–91), p53(Δ364–393), or p53(Gln^{22}-Ser^{23}). The blot was probed with *MCG14* cDNA. *C*, a Northern blot was prepared using 10 μg of total RNA isolated from uninduced cells (−) or cells induced to express (+) wild-type p53, p53(Gln^{22}-Ser^{23}/Gln^{53}-Ser^{54}), or p53(Δ1–42/Gln^{53}-Ser^{54}). The blot was probed with *MCG14* cDNA.

TABLE II
Transcriptional activities of various mutant p53 proteins

	=Fold increase in relative mRNA[a]			
	p21	*mdm2*	*gadd45*	*bax*
Wild-type p53	6.79	40.6	9.45	4.2
p53(Δ1–42)	1.83	8.8	7.03	3.9
p53(Δ62–91)	3.65	6	7	2.7
p53(Gln^{22}-Ser^{23})	1.43	1.2	1.44	1.3

[a] =Fold = mRNA(+p53)/mRNA(−p53).

possibilities is that p53(Gln^{22}-Ser^{23}) might be still able to activate a subset of p53 transcriptional targets which have yet been identified. To this end, we tested the expression patterns of several potential p53 targets identified in our laboratory. We found that one putative p53 transcriptional target, *MCG14*, was activated by p53(Gln^{22}-Ser^{23}) to a level comparable to that by wild-type p53, p53(Δ1–42), p53(Δ64–91), and p53(Δ364–393) (Fig. 4*B*).

Since a double point mutation at residues 22 and 23 abolishes the transcriptional activity of the activation domain I (14), we looked for analogous hydrophobic amino acids within the activation domain II. Two were found: tryptophan at residue 53 and phenylalanine at residue 54. We therefore made identical mutations in these two amino acids in p53(Gln^{22}-Ser^{23}) or p53(Δ1–42), changing tryptophan 53 to glutamine and phenylalanine 54 to serine to generate p53(Gln^{22}-Ser^{23}/Gln^{53}-Ser^{54}) and p53(Δ1–42/Gln^{53}-Ser^{54}). We then established a number of cell lines that inducibly express these mutants, and their ability to induce apoptosis and activate cellular p53 targets were similarly analyzed as above. Three representative cell lines that express either p53(Gln^{22}-Ser^{23}/Gln^{53}-Ser^{54}) or p53(Δ1–42/Gln^{53}-Ser^{54}) are shown in Fig. 5, *A* and *C*, respectively. As expected, Western blot analysis showed that p21 was not activated by either of these mutants (Fig. 5, *A* and *C, bottom panel*). In addition, these mutants were unable to induce apoptosis, as demonstrated by the rate of cell growth (Fig. 5, *B* and *D*), trypan blue exclusion assay, and FACS analysis (Table I). Furthermore, the putative cellular p53 target MCG14, which can be activated by p53(Δ1–42) and p53(Gln^{22}-Ser^{23}) (Fig. 4*B*), failed to be activated in cells expressing either p53(Gln^{22}-Ser^{23}/Gln^{53}-Ser^{54}) or p53(Δ1–42/Gln^{53}-Ser^{54}) (Fig. 4*C*). These results indicate that residues 53 and 54 are critical for the novel domain within residues 43–63 to induce apoptosis and activate cellular p53 targets.

DISCUSSION

The p53 protein has been divided into several functional domains (1, 3, 4) as follows: (*a*) an activation domain which lies within residues 1–42 that has been shown to be required for both transcriptional activation and repression (11, 12, 14, 44); (*b*) a newly identified proline-rich domain within residues 64–91 which is necessary for efficient growth suppression (33), apoptosis (34), and for mediating GAS1-dependent growth arrest (35); (*c*) a sequence-specific DNA-binding domain which lies within the central, conserved portion of the protein (1, 3); (*d*) a nuclear localization signal which lies within residues 316–325 (1, 3); (*e*) a tetramerization domain which lies within residues 334–356 (1, 3); and (*f*) a C-terminal basic domain which binds DNA nonspecifically and regulates the sequence-specific DNA binding activity (1, 3).

Here we found that within residues 43–63 lies another novel domain that is necessary for apoptosis on the basis of the following observations: (i) p53(Δ1–42), which lacks the N-terminal 42 amino acids and the previously defined activation domain, contains a strong apoptotic activity; (ii) p53(Δ1–63), which lacks the N-terminal 63 amino acids but contains intact PXXP motifs, has no apoptotic activity; (iii) a double point mutation at residues 53 and 54 renders both p53(Δ1–42/Gln^{53}-Ser^{54}) and p53(Gln^{22}-Ser^{23}/Gln^{53}-Ser^{54}) completely inert in inducing apoptosis; and (iv) codon 53 is one of the frequently mutated sites outside the DNA binding domain in the *p53* gene in human tumors (45), which underscores the importance of the apoptotic function within residues 43–63 in p53 tumor suppression.

How does this novel domain mediate an apoptotic activity? Previously, it was shown that p53(Gln^{22}-Ser^{23}), which cannot activate several cellular p53 targets (9, 14, 22, 31, 43), is still capable of inducing apoptosis (9, 31, 34), and a p53 mutant, which lacks the proline-rich region, is capable of activating several p53 targets (33, 34) but cannot induce apoptosis (33, 34). These results lead to a hypothesis that p53 has both transcription-dependent and -independent functions in apoptosis. However, it is well established that p53 mutants that are defective in sequence-specific DNA binding activity are also inert in inducing apoptosis (1, 3, 4), suggesting that p53 sequence-specific DNA binding activity and possibly its sequence-specific transcriptional activity are required for inducing apoptosis. Here we found that p53(Δ1–42), which lacks the entire previously defined activation domain I, not only induces apoptosis, but also activates the *MDM2*, *BAX*, and *GADD45* genes through its activation domain II located between residues 43 and 63 (Fig. 4; Table II). Since p53(Gln^{22}-Ser^{23}) contains an

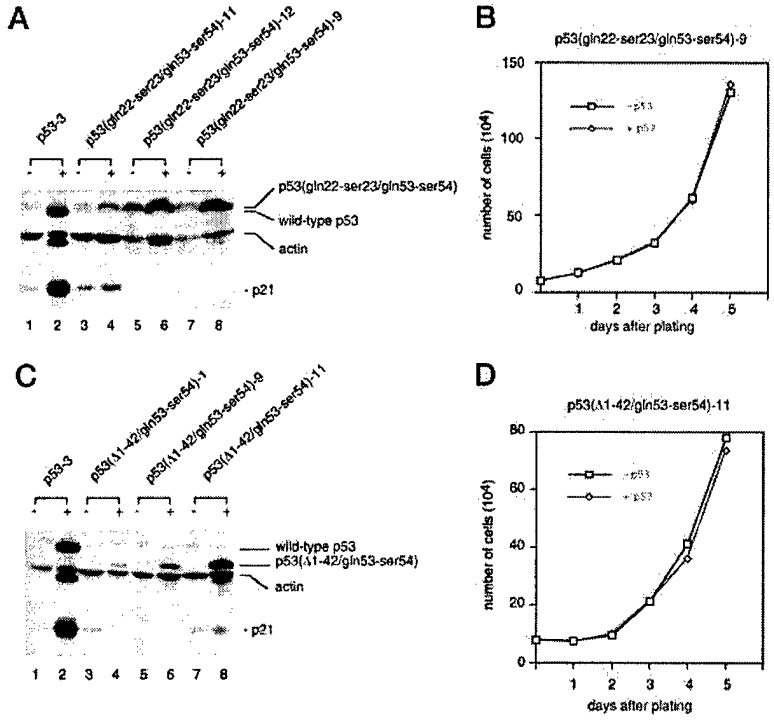

FIG. 5. **A double point mutation at residues 53 and 54 renders both p53(Gln^{22}-Ser^{23}/Gln^{53}-Ser^{54}) and p53(Δ1–42/Gln^{53}-Ser^{54}) completely inert in inducing apoptosis.** *A*, levels of p53, p21, and actin in p53-3, and p53(Gln^{22}-Ser^{23}/Gln^{53}-Ser^{54})-9, -11, and -12 cell lines were assayed by Western blot analysis. *B*, growth rates of p53(Gln^{22}-Ser^{23}/Gln^{53}-Ser^{54})-9 cells in the presence (◇) or absence (□) of p53. *C*, levels of p53, p21 and actin in p53-3, and p53(Δ1–42/Gln^{53}-Ser^{54})-1, -9, and -11 cell lines were assayed by Western blot analysis. *D*, growth rates of p53(Δ1–42/Gln^{53}-Ser^{54})-11 cells in the presence (◇) or absence (□) of p53. The experiments were performed in an identical manner to those in Fig. 1.

intact activation domain II, we hypothesized that it might still contain transcriptional activity. Indeed, we found that p53(Gln^{22}-Ser^{23}) can activate one of the putative p53 targets, *MCG14*. Furthermore, a double point mutation at residues 53 and 54 completely abolishes the ability of both p53(Gln^{22}-Ser^{23}/Gln^{53}-Ser^{54}) and p53(Δ1–42/Gln^{53}-Ser^{54}) to activate MCG14 and induce apoptosis. Consistent with our results, Candau *et al.* (46) recently showed that within residues 40–83 lies a sub-activation domain, which can activate a reporter gene under control of a promoter with a p53-responsive element when p53 is cotransfected, and a double point mutation at residues 53 and 54 also abolished the transcriptional activity of the sub-activation domain. These results suggest that p53 has two independent activation domains. A second activation domain within a transcription factor is not without precedent. Herpes simplex virus protein VP16 also contains two independent activation domains (47). Thus, it appears that in response to various stress conditions and their subsequent modifications, the two independent activation domains might serve as an intrinsic factor of p53 that determines whether a given p53 target is activated. Although *BAX*, *MDM2*, and *GADD45* are the activation domain II-regulated gene products, these cellular p53 targets might not mediate the p53-dependent apoptosis on the basis of two observations: (i) these genes were not activated by p53(Gln^{22}-Ser^{23}) which is competent in inducing apoptosis (Fig. 4*A*; Table II); (ii) these genes were activated by p53(Δ62–91) which is defective in inducing apoptosis (Fig. 4*A*; Table II). Since cell type has been shown to influence the cellular response (cell cycle arrest or apoptosis) to p53 (1, 4, 8), cellular genetic background might then determine the modification of the two activation domains. Therefore, the results obtained in H1299 cells need to be confirmed in other cell types.

It is intriguing that although p53(Gln^{22}-Ser^{23}) contains an intact activation domain II, it fails to activate *BAX*, *GADD45*, and *MDM2* (Fig. 4*A*). Since both p53(Δ1–23) and p53(Δ1–42) can activate these p53 targets, it suggests that the presence of the first 23 amino acids may mask the ability of the activation domain II in p53(Gln^{22}-Ser^{23}) to activate these cellular p53 targets. Alternatively, it is also possible that when the activation domain I is inactivated by a double point mutation at residues 22 and 23, the N-terminal 42 residues might then inhibit or block interaction of a co-activator (or an adaptor) with the activation domain II that is required for activation of some p53 targets, such as *MDM2*, *p21*, *BAX*, and *GADD45*, but not for activation of other p53 targets, such as *MCG14*. It is important to note that although the activation domain I is primarily responsible for activation of p21, the level of p21 in cells expressing either p53(Δ1–23) and p53(Δ1–42) was slightly increased upon p53 induction (Figs. 1*A* and 2*A*), suggesting that the activation domain II can weakly activate p21. Furthermore, our preliminary studies showed that activation of p21 was compromised by a double point mutation at residues 53 and 54 when p53(Gln^{53}-Ser^{54}) was expressed at a low to intermediate level,[3] consistent with the idea that the activation domain II contributes to the activation of p21. Since several clones that express various expression levels of the target genes are required for determining the function of the targets (48), these results remain to be confirmed.

Previously, it was shown that overexpression of p21 can protect human colorectal carcinoma RKO cells from prostaglandin A2-mediated apoptosis (49). Lack of p21 expression due to homologous deletion of the *p21* gene also renders HCT116 colorectal cancer cells susceptible to apoptosis following treatment with either γ-radiation or chemotherapeutic agents (50). In addition, a significant fraction of tumors in mice deriving from $p21^{-/-}$ HCT116 cancer cells were completely cured, and all tumors deriving from $p21^{+/+}$ cancer cells underwent regrowth after treatment with γ-radiation (50). It is interesting to note that p53(Gln^{22}-Ser^{23}) and p53(Δ1–42), both of which lack a functional activation domain I, cannot significantly activate p21 (Fig. 2 and 4; Table II) but can induce apoptosis (Table I). The strong apoptotic activity conferred by p53(Δ1–42) might be due to its failure of activating p21. Thus, we have generated a mutant, p53(Δ1–42), that might be better than wild-type p53 in the elimination of cancer cells and therefore a potential candidate for gene therapy.

[3] J. Zhu, W. Zhou, J. Jiang, and X. Chen,unpublished results.

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Appendix 3

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SHORT REPORT

Differential regulation of cellular target genes by p53 devoid of the PXXP motifs with impaired apoptotic activity

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Activation of the p53 tumor suppressor protein can lead to either cell cycle arrest or apoptosis. Several functional domains necessary for mediating cell cycle arrest and apoptosis in p53 have been mapped, e.g., the proline-rich domain. The proline-rich domain is located within residues 60–90, which comprise five PXXP motifs (where P represents proline and X any amino acid). To further delineate the function of the proline-rich domain and its potential role in transactivation, we generated several groups of cell lines that inducibly express various p53 mutants using a tetracycline-regulated expression system. We found that p53(Δ62–91), which lacks all five PXXP motifs in human p53, is capable of inducing cell cycle arrest but not apoptosis, while p53(gln22-ser23/Δ62–91), which contains a double point mutation in the activation domain as well as deletion of the proline-rich domain, completely loses its activity. However, p53(Δ74–91), which contains only one PXXP motif at its N-terminus, is not only capable of inducing cell cycle arrest but also retains a partial apoptotic activity. Furthermore, we found that deletion of the proline-rich region has no or very mild effects on activation of several transiently transfected p53 target gene promoters, i.e., the p21, MDM2, BAX, and GADD45 promoters. However, such deletion differentially affects p53 induction of endogenous target genes, i.e., induction of p21, MDM2, BTG2, p85, PIG3, PIG6 and PIG11 was reduced or abrogated but induction of BAX, KILLER/DR5, PIG2, PIG7 and PIG8 was not substantially affected. Interestingly, induction of GADD45 was enhanced. These results suggest that the proline-rich region may play a role in chromatin remodeling, which counteracts chromatin-mediated repression for some of the endogenous p53 target genes.

Keywords: p53; p21$^{waf1/cip1}$; apoptosis; cell cycle arrest

The p53 tumor suppressor protein acts as a DNA damage checkpoint and is a pivotal regulator of cellular transformation. Following various genotoxic conditions, p53 accumulates and/or its activity increases, resulting in apoptosis, cell cycle arrest, differentiation (for reviews, see (Agarwal *et al.*, 1998; Ko and Prives, 1996; Levine, 1997) or senescence (Sugrue *et al.*, 1997). As a transcription factor, p53 up-regulates p21$^{waf1/cip1}$, an inhibitor of cyclin-dependent kinases, which is responsible for p53-dependent cell cycle arrest or differentiation (el-Deiry *et al.*, 1993; Harper *et al.*, 1993; Liu *et al.*, 1996; Xiong *et al.*, 1993). However, it is still not certain what exact function p53 plays in apoptosis. Several studies have provided evidence that only certain domains of p53 are required for apoptosis, e.g., the N-terminal 22 amino acids are dispensable (Chen *et al.*, 1996) but the sequence-specific DNA binding domain is required (for reviews, see Gottlieb and Oren, 1996; Ko and Prives, 1996; Levine, 1997). Deletion of the C-terminal regulatory domain reduces p53 apoptotic activity (Chen *et al.*, 1996; Wang *et al.*, 1996), as does a double point mutation at residues 22 and 23 (Chen *et al.*, 1996; Haupt *et al.*, 1995), which also diminishes p53 transcriptional activity (Lin *et al.*, 1994). In addition, several studies have provided evidence that p53 may have a transactivation-independent function in apoptosis (Caelles *et al.*, 1994; Haupt *et al.*, 1995; Wagner *et al.*, 1994).

Recently, the proline-rich region between residues 60 and 90, which contains five 'PXXP' motifs (where P represents proline and X any amino acid), was found to be necessary for efficient growth suppression (Walker and Levine, 1996), serving as a docking site for transactivation-independent growth arrest induced by GAS1 (Ruaro *et al.*, 1997). In addition, the proline-rich region is required for the murine temperature-sensitive p53(val135) to induce apoptosis in adenovirus E1A-transformed cells (Sakamuro *et al.*, 1997).

To further examine the importance of the proline-rich region in human p53, p53(Δ62–91), which lacks all five PXXP motifs, was generated and inducibly expressed in H1299 using the tetracycline-inducible expression system as we have used previously (Chen *et al.*, 1996). H1299 is a p53-null non-small cell lung carcinoma cell line. Nine stable cell lines that inducibly express p53(Δ62–91) were established. Western blot analysis of four cell lines, one expressing wild-type and three expressing mutant p53, is shown in Figure 1a. p53(Δ62–91)-1 is a low p53 producer, and p53(Δ62–91)-5 and -6 are high p53 producers when compared to p53-3, a high wild-type p53 producer cell line established previously (Chen *et al.*, 1996). To determine the transcriptional activity of p53(Δ62–91), we analysed the endogenous p21 gene, a known p53 target (el-Deiry *et al.*, 1993). Western blot analysis demonstrated that p21 was activated in both low and high p53(Δ62–91) producer cells, but the level of induction was approximately 30% of that activated by

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wild-type p53 (Figure 1a). This result suggests that the proline-rich domain may contribute to, but nevertheless is dispensable for, p53 transactivation. To determine whether p53(Δ62–91) can induce cell cycle arrest, the growth rates of two high producer cell lines, p53(Δ62–91)-5 and -6, were analysed. We found that cells expressing p53(Δ62–91) failed to multiply (Figure 1b) or multiply slowly (Figure 1d) while cells not expressing p53(Δ62–91) showed a pattern of exponential growth (Figure 1b and d). FACS analysis showed

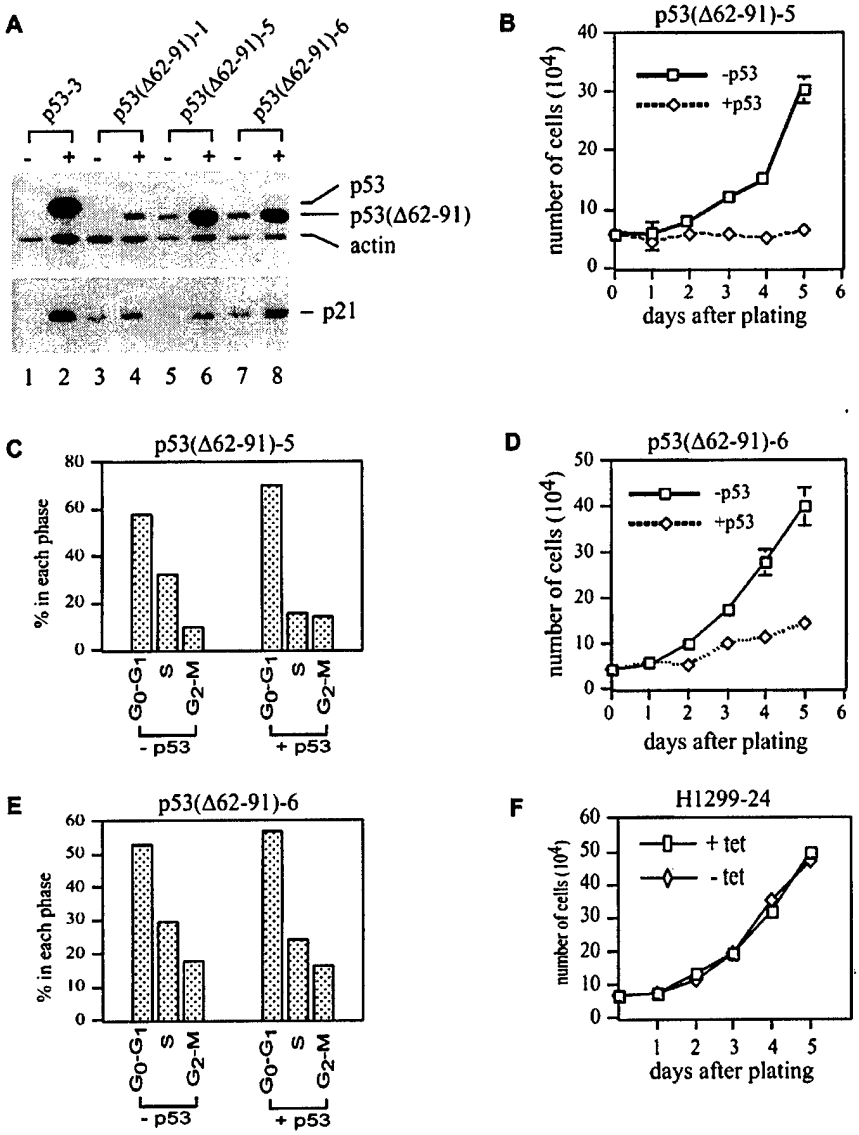

Figure 1 The proline-rich domain is required for p53-dependent apoptosis but not cell cycle arrest. To generate p53(Δ62–91), cDNA fragments encoding amino acids 1-61 and 92-393 were amplified independently and ligated together through an internal *Bam*HI site which was artificially created without affecting the amino acid composition of the p53 protein. Primers used for the amino acids 1–61 fragment were: forward primer N1: GAT CGA ATT CAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC TGA GGA GCC GCA GTC AGA TCC; and reverse primer C61: GAT CGG ATC CGG ACC TGG GTC TTC AGT. Primers used for the amino acid 92-393 fragment were: forward primer N92: GAT CGG ATC CCC TGT CAT CTT CTG TC; and reverse primer C393: GAT CGA ATT CTC AGT CTG AGT CAG GCC CCT. p53(Δ62–91) was then cloned into the tetracycline-regulated expression vector, 10-3, at its *Eco*RI site and the resulting plasmid was used to generate cell lines that inducibly express p53 as previously described (Chen *et al*., 1996). (**a**) Levels of p53, p21, and actin in p53-3, and p53(Δ62–91)-1, -5, and -6 cells were assayed by Western blot analysis. Cell extracts were prepared from uninduced cells (−) or cells induced to express (+) wild-type p53 or p53(Δ62–91). The upper portion of the blot was probed with a mixture of p53 monoclonal antibodies Pab421 and Pab240 and actin polyclonal antibody (Sigma, St Louis, MO, USA). Note that mutant p53(Δ62–91) migrates faster than wild-type p53 because it is missing 30 amino acids. The lower portion of the blot was probed with p21 monoclonal antibody (Oncogene Science, Uniondale, NY, USA). (**b** and **d**) Growth rates of p53(Δ62–91)-5 and -6 cells in the presence (◇) or absence (□) of p53 were measured as previously described (Zhu *et al*., 1998). (**c** and **e**) The percentages of p53(Δ62–91)-5 and -6 cells in G_0-G_1, S, and G_2-M phases in the presence or absence of p53 for 3 days were quantitated by FACS analysis as described previously (Zhu *et al*., 1998). (**f**) Growth rates of H1299-24 cells in the presence (+tet) or absence (−tet) of tetracycline. The experiments were performed similarly as in (**b**)

that the number of S-phase cells was markedly reduced in p53(Δ62–91)-5 cells and moderately reduced in p53(Δ62–91)-6 cells upon induction of p53 (Figure 1c and e). Thus, both growth rate and FACS analyses indicate that the proline-rich domain in p53 is not required for cell cycle arrest. As control, the growth rates of H1299-24 cell line was similarly established but does not express any protein were nearly identical in the presence (□ + tet) or absence (◇-tet) of tetracycline (Figure 1f).

It is well established that p53 can induce apoptosis in H1299 cells (Chen *et al.*, 1996; Haupt *et al.*, 1995). This can be quantitated by determining the percentage of cells containing a sub-G_1 content of DNA. FACS analysis showed that only 2–6% of cells underwent apoptosis after induction of p53(Δ62–91) for 3 days as determined from three separate experiments. Using trypan blue exclusion assay (Zhu *et al.*, 1998), a similar percentage of dead cells were detected. In contrast, about 45% and 30% of cells underwent apoptosis after

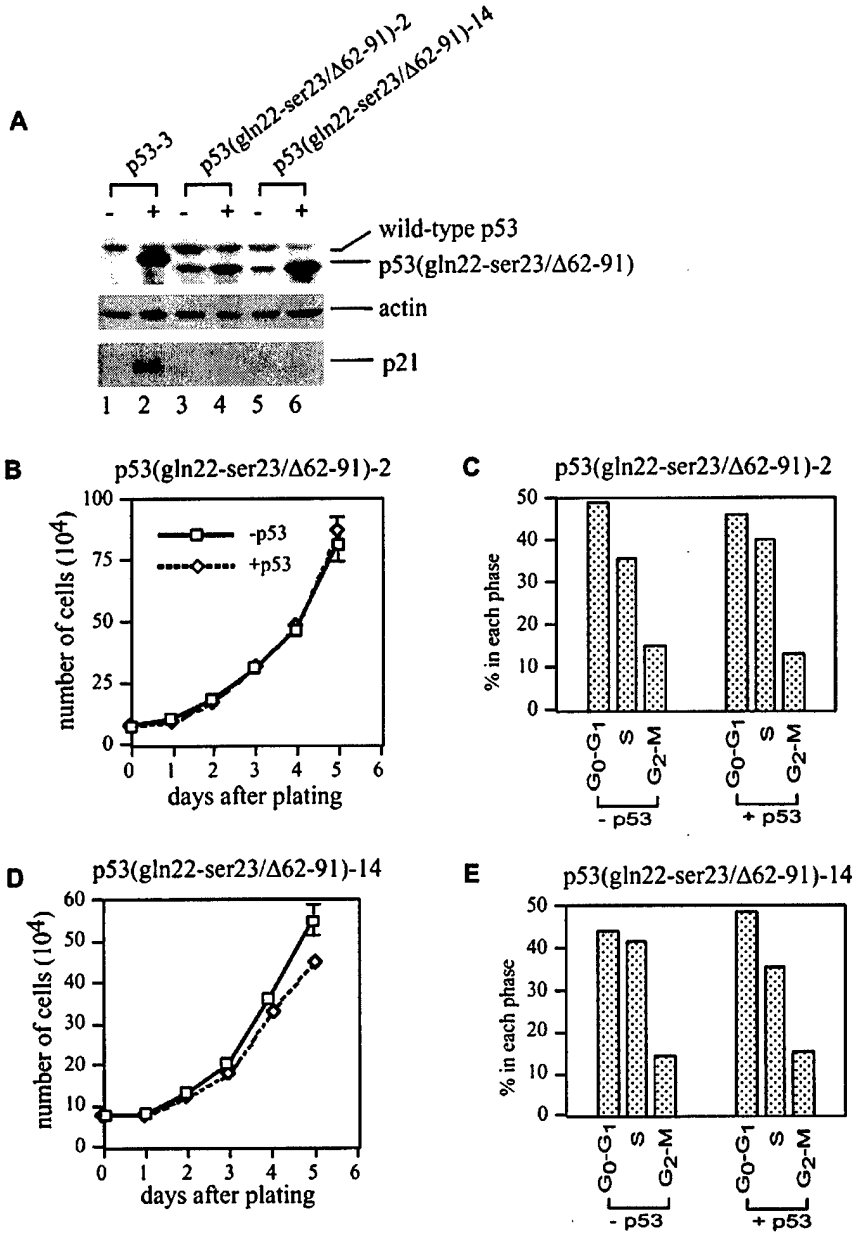

Figure 2 Deletion of the proline-rich domain abolished the apoptotic activity of the transactivation deficient p53(gln22-ser23). p53(gln22-ser23/Δ62–91) was generated similarly as p53(Δ62–91) except that a cDNA encoding p53(gln22-ser23) (Lin *et al.*, 1994) was used as template. (**a**) Levels of p53, p21 and actin in p53-3 and p53(gln22-ser23/Δ62–91)-2 and -14 cells were assayed by Western blot analysis. (**b** and **d**) Growth rates of p53(gln22-ser23Δ62–91)-2 and -14 cells in the presence (◇) or absence (□) of p53. (**c** and **e**) The percentage of p53(gln22-ser23Δ62–91)-2 and -14 cells in G_0-G_1, S, and G_2-M phases in the presence or absence of p53 for 3 days. The experiments were performed in an identical manner to those shown in Figure 1

induction of wild-type p53 and transactivation deficient p53(gln22-ser23), respectively.

Previously, we and others have shown that p53(gln22-ser23) retains a partial apoptotic activity but is incapable of inducing cell cycle arrest (Chen *et al.*, 1996; Haupt *et al.*, 1995). To further demonstrate the function of the proline-rich region, we hypothesized that p53(gln22-ser23/Δ62–91), which contains a double point mutation at residues 22 and 23 in the activation domain as well as deletion of the proline-rich domain, would be unable to induce either cell cycle arrest or apoptosis. Four stable cell lines that inducibly express p53(gln22-ser23/Δ62–91) were established and two representative cell lines were characterized (Figure 2a). We found that this p53 mutant is unable to activate p21 (Figure 2a). Furthermore, growth rate (Figure 2b and d) and FACS (Figure 2c and e) analyses showed that neither cell cycle arrest nor apoptosis were observed in cells expressing p53(gln22-ser23/Δ62–91) under the induced condition. These

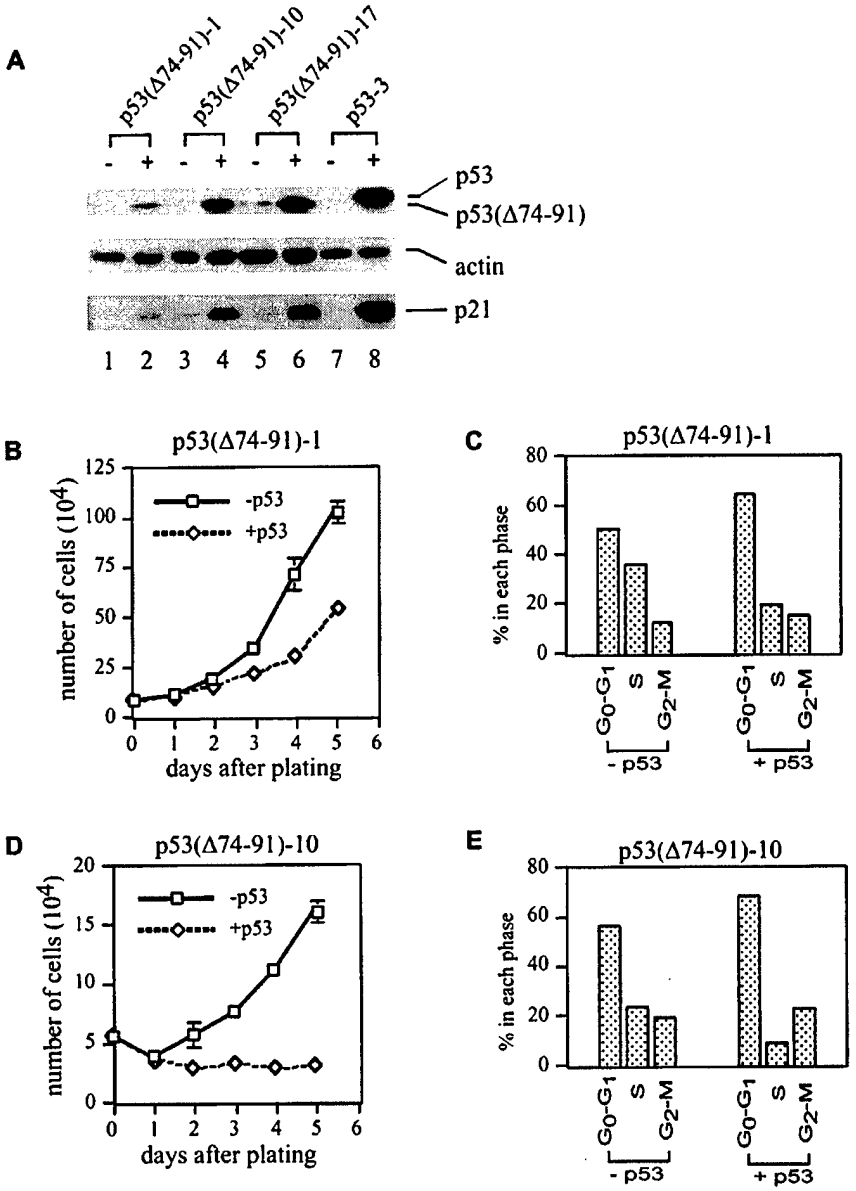

Figure 3 The N-terminal PXXP motif is necessary for mediating apoptosis. To generate p53(Δ74–91), cDNA fragments encoding amino acids 1–73 and 92–393 were amplified independently and ligated together through an internal *KpnI* site which was artificially created without affecting the amino acid composition of the p53 protein. Primers used for the amino acid 1–73 fragment were: forward primer N1 as used for generating p53(Δ62–91); and reverse primer C73: GAT CGG TAC CGG GGG AGC AGC CTC TGG. Primers used for the amino acids 92–393 fragment were: forward primer N92-1: GAT CGG TAC CCC TGT CAT CTT CTG TC; and reverse primer C393 as used for generating p53(Δ62–91). (**a**) Levels of p53, p21, and actin in p53-3, and p53(Δ74–91)-1, -10, and -17 cells were assayed by Western blot analysis. (**b** and **d**) Growth rates of p53(Δ74–91)-1 and -10 cells in the presence (◇) or absence (□) of p53. (**c** and **e**) The percentage of p53(Δ74–91)-1 and -10 cells in G_0-G_1, S, and G_2-M phases in the presence or absence of p53 for 3 days. The experiments were performed in an identical manner to those shown in Figure 1

results indicate that the apoptotic activity of p53(gln22-ser23) is lost upon deletion of the proline-rich region.

While the human p53 protein contains five PXXP motifs, the rat and murine contain only one and two, respectively (Walker and Levine, 1996). Thus, we investigated whether the dosage of the PXXP motifs can determine the extent of apoptotic response. To this end, we generated p53(Δ74–91), which lacks the C-terminal four PXXP motifs. Ten stable cell lines were established that inducibly express this mutant and three representative cell lines were characterized (Figure 3a). p53(Δ74–91)-1 is a low p53 producer while p53(Δ74–91)-10 and -17 are high p53 producers. Western blot analysis showed that p21 was moderately activated by p53(Δ74–91), to approximately 50% of that activated by wild-type p53 (Figure 3a). Nevertheless, this mutant can efficiently induce cell cycle arrest as measured by the rate of cell growth (Figure 3b and d) or by the reduction of cells in S phase (Figure 3c and e). In addition, we found that 10–17% of cells underwent apoptosis as detected by FACS analysis. These results indicate that p53(Δ74–91) retains a partial apoptotic activity.

Previously, it was shown that the p21 promoter can be activated by a PXXP deletion mutant to an extent that is equal to or greater than by wild-type p53 (Venot *et al.*, 1998; Walker and Levine, 1996). A similar result was observed when we tested the ability of p53(Δ62–91) to activate the p21 promoter in a luciferase assay (data not shown). However, Western blot analysis consistently showed that p53(Δ62–91) is much weaker than wild-type p53 to induce p21 (Figure 1a; data not shown). To determine whether the low level of p21 protein detected in p53(Δ62–91)-expressing cells is due to decreased expression of the p21 gene, we performed Northern blot analysis. We found that p21 was induced by p53(Δ62–91) but the extent of induction was approximately 30% of that by wild-type p53 (Figure 4a; Table 1). As controls, we used two previously characterized cell lines, p53(gln22-ser23)-2 and p53(R249S)-4 (Chen *et al.*, 1996), which inducibly express the transactivation deficient p53(gln22-ser23)

Table 1 Transcriptional activities of various mutant p53 proteins

	= Fold increase in relative mRNA[a]			
	wild-type p53	*p53 (Δ62–91)*	*p53 (gln22–ser23)*	*p53 (R249S)*
p21	11.1	3.8	1.5	1.2
MDM2	6.6	2.4	1.1	1.2
GADD45	3	5.5	1.2	1.1
BAX	3.5	2.9	1.4	1.0
BTG2	8.2	2.5	ND[b]	ND
p85	2.8	1.5	1.2	1.0
KILLER/DR5	2.7	3.1	1.3	1.1
PIG2	2.5	2.1	1.3	1.0
PIG3	30	2.3	1.1	1.2
PIG6	9.9	ND	ND	ND
PIG7	3.1	3.7	1.2	1.1
PIG8	3.5	3.0	1.2	1.1
PIG11	15.4	ND	ND	ND

[a]Fold = mRNA(+p53)/mRNA(−p53); [b]ND = not done

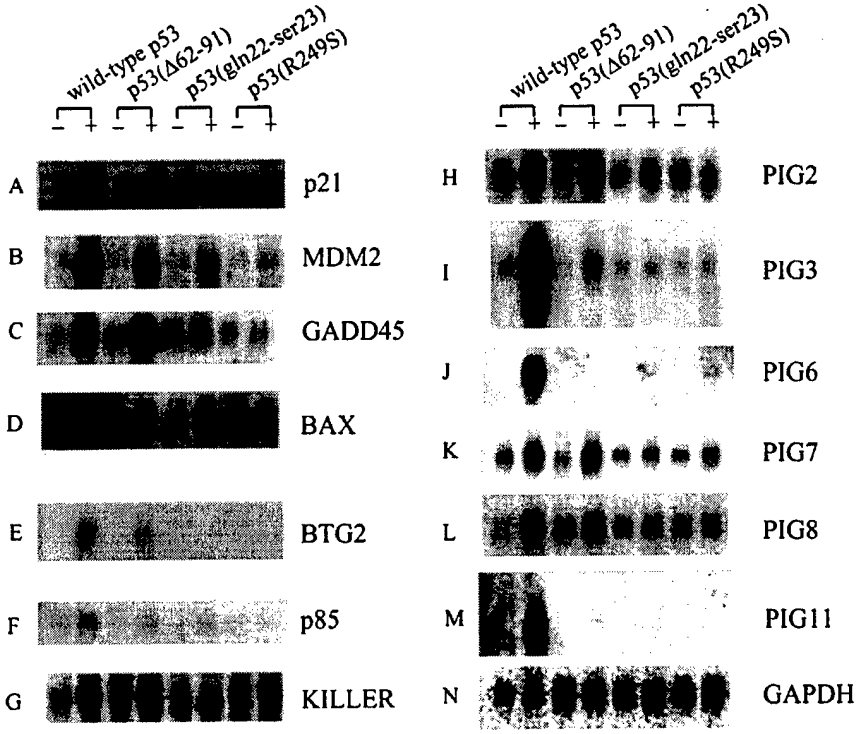

Figure 4 Deletion of the PXXP motifs differentially affects p53 induction of endogenous target genes. Northern blots were prepared using 10 μg of total RNA isolated from uninduced cells (−) or cells induced to express (+) wild-type p53, p53(Δ62–91), p53(gln22-ser23) and p53(R249S). Northern blot analysis was performed as described (Zhu *et al.*, 1998). The p21, MDM2, BAX, GADD45 and GADPH probes were prepared as described (Zhu *et al.*, 1998). The KILLER/DR5 cDNA probe (GenBank #159553) was purchased from American Type Culture Collection. The following cDNA probes were purchased from Genome System, Inc (St. Louis, MO, USA): BTG2 (GenBank #H86711), PIG1 (W61024), PIG2 (H18355), PIG3 (N75824), PIG4 (H45773), PIG6 (R88591), PIG8 (R42786), PIG10 (R87338), PIG11 (R54648), PIG12 (AA149234) and p85 (N21330)

and tumor-derived mtuant p53(R249S), respectively. Both p53(gln22-ser23) and p53(R249S) were inert in inducing p21 (Figure 4a; also see Table 1).

Next, we determined whether p53(Δ62–91) can regulate other p53 target genes, i.e., MDM2, GADD45, BAX, BTG2, p85 and KILLER/DR5. We found that MDM2, an oncogene and a negative regulator of p53 (Wu *et al.*, 1993), was slightly induced by p53(Δ62–91) (Figure 4b; Table 1). However, GADD45, a DNA damage responsive gene involved in DNA repair and growth suppression (Kastan *et al.*, 1992), was substantially induced by p53(Δ62–91) to an extent that is nearly two times more than by wild-type p53 (Figure 4c; Table 1). It is well known that p21 is primarily responsible for p53-dependent cell cycle arrest. Thus, although the induction of p21 is compromised by deletion of the PXXP motifs, it is possible that an enhanced induction of GADD45 may compensate the decreased induction of p21, resulting in cell cycle arrest detected in p53(Δ62–91)-expressing cells (Figure 1). BAX, an apoptosis activator, can be weakly induced by wild-type p53 (Miyashita *et al.*, 1994) as shown in this study (Figure 4d; Table 1). However, BAX expression was only slightly reduced by deletion of the PXXP motifs (Figure 4d; Table 1). BTG2, a nerve growth factor responsive gene that can cause growth suppression (Rouault *et al.*, 1996), was strongly induced by p53 but only slightly by p53(Δ62–91) (Figure 4e; Table 1). p85, a regulatory subunit of the signaling protein phosphatidyl-3-OH kinase (PI(3)K), was shown to be involved in the p53-dependent apoptotic response to oxidative stress (Yin *et al.*, 1998). Upon H_2O_2 treatment, the level of p85 was increased in a p53-dependent manner (Yin *et al.*, 1998). We found that p85 was induced 2–3-fold by wild-type p53 but little if any by p53(Δ62–91) (Figure 4f; Table 1). KILLER/DR5, a death receptor gene which can be induced by genotoxic stress and p53 (Wu *et al.*, 1997), was similarly induced by both wild-type p53 and p53(Δ62–91) (Figure 4g; Table 1).

Several redox-related genes (PIGs), that were shown to be activated by p53 and potentially involved in p53-dependent apoptotic pathway (Polyak *et al.*, 1997), were examined for p53(Δ62–91) induction. We confirmed that PIG2, PIG3, PIG6, PIG7, PIG8 and PIG11 were significantly induced by wild-type p53 in H1299 cells (Figure 4h–m; Table 1). We found that induction of PIG2, PIG7, and PIG8 was not significantly affected by deletion of the proline-rich region (Figure 4h, k and m; Table 1) but induction of PIG3, PIG6 and PIG11 was substantially reduced or abrogated (Figures 3i, 4j and 4m; Table 1). We also found that PIG10 and PIG12 were not substantially induced by p53, and PIG1 and PIG4 were undetectable in H1299 cells (data not shown). Therefore, p53(Δ62–91) induction of the PIG1, PIG4, PIG10 and PIG12 genes was not analysed.

Our studies have confirmed and extended the previous observation (Walker and Levine, 1996) that the proline-rich domain in human p53 is necessary for efficient growth suppression. Specifically, we found that the proline-rich domain is required for p53-dependent apoptosis. Thus, this function is conserved between human and murine p53 (Sakamura *et al.*, 1997). We also found that only the N-terminal PXXP motif is required for mediating apoptosis, indicating that the proline-rich domain contains redundant effector PXXP components. Furthermore, we have shown that the proline-rich domain can differentially regulate p53 induction of endogenous target genes: deletion of the proline-rich domain has no significant effects on induction of Bax, KILLER/DR5, PIG2, PIG7 and PIG8; however, induction of p21, MDM2, BTG2, p85, PIG3, PIG6, and PIG11 were substantially reduced or abrogated. Since p85 is involved in the apoptotic response to oxidative stress (Yin *et al.*, 1998) and PIG3 and PIG6 can produce reactive oxygen species leading to degradation of mitochondria and subsequently apoptosis (Polyak *et al.*, 1997), the results suggest that the PXXP motifs in p53 are required for activating genes that participate directly in signaling pathways controlling apoptosis.

It should be noted that the differential regulation of cellular target genes by p53(Δ62–91) may not be uncovered if its transcriptional activity was measured by its capability of activating the p21 or other target gene promoters in a transient transfection assay. In addition to the p21 promoter, the MDM2, GADD45, and BAX promoters can be activated by p53 lacking the proline-rich domain as efficiently as wild-type p53 (Venot *et al.*, 1998; data not shown). Thus, we should not extrapolate the result observed by the promoter analysis of a target gene to p53 induction of the endogenous target gene. It is well established that the regulation of transcription for endogenous genes that are packaged into chromatin is different from those that are transiently transfected into cells in naked plasmids (Smith and Hager, 1997). The simplified explanation is that the promoter template in a naked plasmid would be easily accessible while that packaged into chromatin is not. It is well known that transcriptional activators function, at least in part, to counteract chromatin-mediated repression (Kadonaga, 1998). For example, MyoD, a basic helix–loop–helix transcriptional activator, contains two domains that are necessary for chromatin remodeling but are not associated with any known activation function (Gerber *et al.*, 1997). While deletion of these two domains lead to very mild effects on activation of transiently transfected templates, activation of endogenous templates depends on these two domains of MyoD. Since the PXXP motifs in p53 have a function similar to these two domains in MyoD in differentially activating transfected and endogenous templates, it suggests that the proline-rich region may be necessary for chromatin remodeling, which is responsible for p53 as transcriptional activator to counteract chromatin-mediated repression for some of the cellular p53 target genes, resulting in their differential induction.

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Appendix 4

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Definition of the p53 Functional Domains Necessary for Inducing Apoptosis*

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The p53 protein contains several functional domains necessary for inducing cell cycle arrest and apoptosis. The C-terminal basic domain within residues 364–393 and the proline-rich domain within residues 64–91 are required for apoptotic activity. In addition, activation domain 2 within residues 43–63 is necessary for apoptotic activity when the N-terminal activation domain 1 within residues 1–42 is deleted (ΔAD1) or mutated (AD1$^-$). Here we have discovered that an activation domain 2 mutation at residues 53–54 (AD2$^-$) abrogates the apoptotic activity but has no significant effect on cell cycle arrest. We have also found that p53-(ΔAD2), which lacks activation domain 2, is inert in inducing apoptosis. p53-(AD2$^-$ΔBD), which is defective in activation domain 2 and lacks the C-terminal basic domain, p53-(ΔAD2ΔBD), which lacks both activation domain 2 and the C-terminal basic domain, and p53-(ΔPRDΔBD), which lacks both the proline-rich domain and the C-terminal basic domain, are also inert in inducing apoptosis. All four mutants are still capable of inducing cell cycle arrest, albeit to a lesser extent than wild-type p53. Interestingly, we have found that deletion of the N-terminal activation domain 1 alleviates the requirement of the C-terminal basic domain for apoptotic activity. Thus, we have generated a small but potent p53-(ΔAD1ΔBD) molecule. Furthermore, we have determined that at least two of the three domains (activation domain 1, activation domain 2, and the proline-rich domain), are required for inducing cell cycle arrest. Taken together, our results suggest that activation domain 2 and the proline-rich domain form an activation domain for inducing pro-apoptotic genes or inhibiting anti-apoptotic genes. The C-terminal basic domain is required for maintaining this activation domain competent for transactivation or transrepression.

Activation of p53 leads to at least two well defined cellular responses: cell cycle arrest and apoptosis (1–4). Based on these activities and other characteristics (1, 5), the p53 protein can be divided into several functional domains. These are activation domain 1 within residues 1–42 (6–8), activation domain 2 within residues 43–63 (9–11), the proline-rich domain within residues 64–91 (12), the sequence-specific DNA-binding domain within residues 100–300 (1), the nuclear localization signal within residues 316–325 (13), the tetramerization domain within residues 334–356 (14), which also contains a nuclear export signal (15), and the C-terminal basic domain within residues 364–393 (1, 5).

p53 is frequently mutated in cancers. Mutations in the p53 DNA binding domain or certain mutations in the nuclear localization signal and tetramerization domain that indirectly affect DNA binding abrogate or diminish p53 activity in cell cycle arrest and apoptosis (1, 5). The proline-rich domain has been shown to be required for efficient growth suppression (12). Recent experiments indicate that the proline-rich domain is necessary for apoptosis but not cell cycle arrest (16–18). In addition, the proline-rich domain plays an important role in the induction of several endogenous target genes, but is not required for activation of the exogenously introduced promoters of these target genes (17). These results suggest that the proline-rich domain may participate in the induction of cellular target gene(s) responsible for mediating apoptosis. However, the role of other p53 functional domains (especially the N-terminal activation domain 1 and the C-terminal basic domain) in apoptosis is still not certain. Earlier reports have shown that in some experimental protocols (19–21) including our own (22), p53 transactivation activity is dispensable for apoptosis. It should be noted that this conclusion is based at least in part on the observation that an activation domain 1-deficient mutant (a double point mutation at residues 22–23, AD1$^-$)[1] is capable of inducing apoptosis (21, 22). Recently, we and others have shown that p53-(AD1$^-$) contains an intact activation domain 2 (9–11), and therefore, p53-(AD1$^-$) is still competent in transactivation (10). Furthermore, when both activation domain 1 and activation domain 2 are mutated (a quadruple point mutation at residues 22–23 and 53–54, AD1$^-$AD2$^-$), the resulting protein is inert in transactivation and in inducing cell cycle arrest and apoptosis (9–11).

The C-terminal basic domain has been subjected to extensive analysis, and all evidence suggests that the basic domain is a regulatory domain. This basic domain can regulate the DNA binding activity when it is phosphorylated (1, 5), acetylated (23–25), deleted (26), or associated with anti-p53 antibody (26, 27) or peptides derived from the C terminus of p53 (28, 29). Interestingly, the mechanism by which these latter peptides enhance p53 DNA binding activity is the ability of the peptides to interact with three separate domains in p53, that is, the proline-rich domain (30), the DNA binding domain (31), and the C-terminal basic domain (30, 31). The C-terminal basic domain also interacts with several cellular proteins, such as TFIIH subunits XPB and XPD (32, 33), and Werner syndrome protein (WRN) (34, 35), which all lead to efficient induction of p53-mediated apoptosis. These results support a hypothesis that the C-terminal basic domain is a negative regulatory do-

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[1] The abbreviations used are: AD, activation domain; HA, hemagglutinin; PRD, proline-rich domain; BD, C-terminal basic domain.

main whose effect on the DNA binding activity can be alleviated by interacting with other cellular proteins, peptides derived from the p53 C terminus, or other modifications. However, several groups have shown that p53-(ΔBD), which lacks the C-terminal basic domain, has a reduced ability to induce several cellular target genes and becomes incapable of inducing apoptosis (22, 32, 36). These results suggest that the C-terminal basic domain can regulate p53 activity both positively and negatively.

In this study, we show that activation domain 2 and the proline-rich domain form an activation domain for inducing pro-apoptotic genes or inhibiting anti-apoptotic genes. The C-terminal basic domain is required for maintaining this activation domain competent for transactivation or transrepression. We also found that an activation domain capable of inducing at least partial cell cycle arrest can be formed by activation domain 1 plus activation domain 2, activation domain 1 plus the proline-rich domain, or activation domain 2 plus the proline-rich domain. The ability of these activation domains to induce cell cycle arrest can be enhanced by the presence of the C-terminal basic domain.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis—Mutant p53 cDNA constructs were generated by polymerase chain reaction, and mutations were confirmed by DNA sequencing. All p53 proteins were tagged at their N termini with an influenza hemagglutinin (HA) peptide recognizable by anti-HA antibody 12CA5. HA-tagged wild-type p53 was generated using 5′-end primer 5HA (GATCGAATTCACCATGGGCTACCCATACGATGTTCCAGATTACGCTGAGGAGCCGCAGTCAGATCC) and 3′-end primer C393 (GATCGAATTCTCAGTCTGAGTCAGGCCCTT). To generate p53-(AD2$^-$), cDNA fragments encoding amino acids 1–59 and 60–393 were amplified independently and ligated through an internal *Ava*II site. The cDNA fragment encoding amino acids 1–59 was amplified by 5′-end primer 5HA and 3′-end primer C59 (TTCATCTGGACCTGGGTCTTCAGTGCTCTGTTGTTCAATATC). The cDNA fragment encoding amino acids 60–393 was amplified by 5′-end primer N60 (ACTGAAGACCCAGGTCCA) and 3′-end primer C393. To generate p53-(ΔAD2), a cDNA fragment that encodes residues 41–393 but lacks residues 43–63 was amplified by 5′-end primer AD2 (TTGCAATGGATGATGCTCCCAGAATGCCAGA) and 3′-end primer C393. This fragment was then used to replace the HA-tagged wild-type p53 from residues 41–393 at a *Bsr*D1 site. To generate p53-(ΔAD2ΔPRD), a cDNA fragment that encodes residues 41–393 but lacks residues 43–91 was amplified by 5′-end primer AP5 (TTGCAATGGATGATCCCCTGTCGTCTTCTGT) and 3′-end primer C393. This fragment was then used to replace the HA-tagged wild-type p53 from residues 41–393 at a *Bsr*D1 site. p53-(ΔAD1), p53-(ΔPRD), p53-(ΔBD), p53-(ΔAD1AD2$^-$), and p53-(ΔAD1ΔAD2) were generated as described previously (10, 17, 22). To generate p53-(ΔAD1ΔPRD), p53-(ΔPRD) cDNA was amplified by 5′-end primer N43 (GATCGAATTCACCATGGGCTACCCATACGATGTTCCAGATTACGCTTTGATGCTGTCCCCG) and 3′-end primer C393. To generate p53-(AD2$^-$ΔBD), p53-(ΔAD2ΔBD), p53-(ΔPRDΔBD), p53-(ΔAD1ΔBD), p53-(ΔAD1AD2$^-$ΔBD), p53-(ΔAD1ΔPRDΔBD), p53-(ΔAD1ΔAD2ΔBD), and p53-(ΔAD2ΔPRDΔBD), the 3′-end cDNA fragments starting from the *Stu*I site in p53-(AD2$^-$), p53-(ΔAD2), p53-(ΔPRD), p53-(ΔAD1), p53-(ΔAD1AD2$^-$), p53-(ΔAD1ΔPRD), p53-(ΔAD1ΔAD2), and p53-(ΔAD2ΔPRD) were replaced with the corresponding cDNA fragment in p53-(ΔBD).

The above mutant p53 cDNAs were cloned separately into a tetracycline-regulated expression vector, pUHD10-3, at its *Eco*RI site (37), and the resulting plasmids were used to generate cell lines that inducibly express p53.

Cell Lines—H1299 and MCF7 cell lines that express inducible proteins of interest were generated as described previously (10, 17, 22). The H1299 cell lines p53-3, p53-(R249S)-4, p53-(AD1$^-$)-2, p53-(ΔBD)-1, p53-(ΔPRD)-5, and p53-(ΔAD1)-2 were as described previously (10, 17, 22).

Western Blot Analysis—Western blot analysis was performed as described (10, 17, 22), with anti-p53 monoclonal antibody Pab240, anti-HA monoclonal antibody 12CA5 (Roche Molecular Biochemicals), anti-actin polyclonal antibody (Sigma), and anti-p21 monoclonal antibody (Ab-1) (Oncogene Research Products, Cambridge, MA).

Growth Rate Analysis, Trypan Blue Dye Exclusion Assay, DNA Histogram Analysis, and Annexin V Staining—Growth rate analysis, trypan blue dye exclusion assay, and DNA histogram analysis were performed as described previously (10, 17, 22). Propidium iodide and RNase A were purchased from Sigma. Fluorescein isothiocyanate-labeled annexin V was purchased from Roche Molecular Biochemicals, and staining was performed as described by the manufacturer.

RNA Isolation and Northern Blot Analysis—Total RNA was isolated using Trizol reagents (Life Technologies, Inc.). Northern blot analysis was performed as described (10). The *p21*, *BAX*, and glyceraldehyde-3-phosphate dehydrogenase probes were prepared as described previously (10).

RESULTS

The Activity of Activation Domain 2 Is Necessary for Inducing Apoptosis—Previously, we have shown that the activity of activation domain 2 is required for inducing apoptosis when a double point mutation at residues 22–23 or deletion of the N-terminal 42 amino acid residues renders activation domain 1 dysfunctional (10). To further determine the function of activation domain 2 in apoptosis, we generated an activation domain 2-deficient mutant, p53-(AD2$^-$), which contains a double point mutation at residues 53–54. We then established several cell lines that inducibly express this mutant in *p53*-null H1299 lung carcinoma cells. Western blots from two representative cell lines, p53-(AD2$^-$)-6 and -8, are shown in Fig. 1*A*. After normalization to the levels of actin protein expressed, we found that the levels of p53 protein in p53-(AD2$^-$)-6 and -8 cells were comparable with that in p53-3 and HA-p53-15 cells, which express wild-type p53 and HA-tagged wild-type p53, respectively (Fig. 1*A*, *upper two panels*, compare *lanes 5–8* with *lanes 1–4*). To determine the transcriptional activity of p53-(AD2$^-$), we measured the level of p21 protein induced by p53-(AD2$^-$). Surprisingly, we found that the ability of p53-(AD2$^-$) to induce *p21* was severely diminished (Fig. 1*A*, *p21 panel*, *lanes 5–8*). These results are similar to that observed for the activation domain 1-deficient mutant (6, 10, 22). In contrast, *p21* was strongly induced by wild-type p53 and HA-tagged wild-type p53 (Fig. 1*A*, *p21 panel*, *lanes 1–4*).

One of the hallmarks for p53 when overexpressed in cells is growth suppression (1–3). The HA-tagged wild-type p53 protein in HA-p53-15 cells, like the untagged wild-type p53 in p53-3 cells (10, 22), inhibits cell proliferation (data not shown). To determine the activity of p53-(AD2$^-$) in H1299 cells, the growth rate of p53-(AD2$^-$)-6 cells was determined over a 5-day period. When induced to express p53-(AD2$^-$), cells failed to multiply (Fig. 1*B*), but visible microscopic cell death was not significantly increased (data not shown).

Previously, several studies have shown that the C-terminal basic domain is necessary for inducing apoptosis but not cell cycle arrest (22, 32). To determine whether this domain has any effect on the ability of p53-(AD2$^-$) to induce growth suppression, we generated p53-(AD2$^-$ΔBD), which is deficient in activation domain 2 and has a deletion of the C-terminal basic domain. We then established several cell lines that inducibly express p53-(AD2$^-$ΔBD). Western blots from three representative cell lines, p53-(AD2$^-$ΔBD)-2, -8, and -9, are shown in Fig. 1*A*. We found that the levels of p53 in these cells were comparable with that in HA-p53-15 and p53-(ΔBD)-1 cells (Fig. 1*A*, *upper two panels*, compare *lanes 3–4* and 9–16). p53-(ΔBD)-1 cells are derived from H1299 cells that inducibly express p53-(ΔBD), which lacks the C-terminal basic domain (22). Similarly, the transcriptional activity of p53-(AD2$^-$ΔBD) was determined by measuring the level of *p21* induced. We found that, like p53-(AD2$^-$), the ability of p53-(AD2$^-$ΔBD) to induce *p21* was significantly diminished (Fig. 1*A*, *p21 panel*, compare *lanes 11–16* with *lanes 1–4*). In contrast, *p21* was strongly induced by p53-(ΔBD) (Fig. 1*A*, *p21 panel*, *lanes 9–10*), consistent with previous reports (22, 32). Nevertheless, growth rate analysis showed that p53-(AD2$^-$ΔBD) was still capable of inhibiting cell

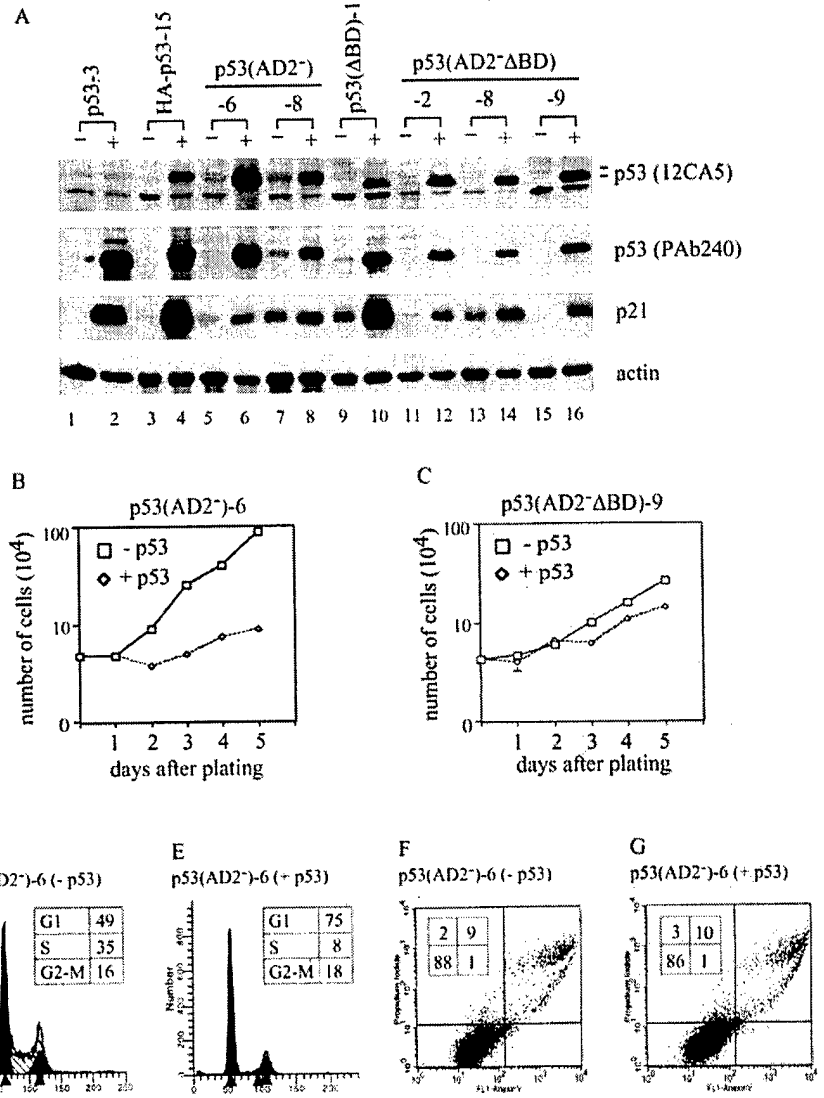

FIG. 1. **The activity of activation domain 2 is necessary for inducing apoptosis.** *A*, levels of p53, p21, and actin were assayed by Western blot analysis in cell lines as shown above the blots. Cell extracts were prepared from non-induced cells (−) and cells induced to express p53 for 24 h (+). HA-tagged p53 was detected with 12CA5 antibody. p53 was detected with anti-p53 monoclonal antibody Pab240. p21 was detected with anti-p21 monoclonal antibody (Ab-1). Actin was detected with anti-actin polyclonal antibody. *B* and *C*, growth rates of p53-(AD2⁻)-6 and p53-(AD2⁻ΔBD)-9 cells in the absence (◇) or presence (□) of p53 over a 5-day period. *D* and *E*, DNA content was quantified by propidium iodide staining of fixed cells that were non-induced (− p53) or induced (+ p53) to express p53-(AD2⁻) for 3 days. *F* and *G*, apoptotic cells were quantified by propidium iodide-annexin V staining of cells that were non-induced (− p53) or induced (+ p53) to express p53-(AD2⁻) for 3 days.

growth (Fig. 1*C*), albeit to a lesser extent than p53-(AD2⁻) (Fig. 1*B*).

To determine whether the growth suppression by p53-(AD2−) is because of cell cycle arrest, apoptosis, and/or both, we performed a DNA histogram analysis and an annexin V staining assay. When induced to express the mutant *p53-*(AD2⁻) for three days, we found that the percentage of cells in S phase decreased from 35 to 8% whereas cells in G_1 increased from 49 to 75%, suggesting that p53-(AD2⁻) arrested cells primarily in G_1 (Fig. 1, *D–E*). However, no apparent apoptosis was detected by either DNA histogram analysis (Fig. 1, *D–E*) or annexin V staining (Fig. 1, *F–G*). Thus, the activity in activation domain 2 is necessary for inducing apoptosis. As a positive control, we analyzed p53-3 and HA·p53-15 cells. When induced to express wild-type or HA-tagged p53 for three days, we found that both p53-producing cells were arrested primarily in G_1 and underwent apoptosis, consistent with previous reports (10, 22). We also analyzed p53-(AD2⁻ΔBD)-9 cells. We found that no significant apoptosis was observed, and cells primarily arrested in G_1 when induced to express p53-(AD2⁻ΔBD) (data not shown).

To determine the activity of the entire activation domain 2 (residues 43–62), we generated p53-(ΔAD2), which lacks the entire activation domain 2 and p53-(ΔAD2ΔBD), which in turn lacks activation domain 2 and the C-terminal basic domain. We then established several cell lines that inducibly express p53-(ΔAD2) and p53-(ΔAD2ΔBD), respectively (Fig. 2, *A* and *C*). We found that p53-(ΔAD2) and p53-(ΔAD2ΔBD) suppressed cell proliferation (Fig. 2, *B* and *D*), albeit to a lesser extent than p53-(AD2⁻) and p53-(AD2⁻ΔBD) (Fig. 1, *B* and *C*). Furthermore, we found that cells were arrested primarily in G_1 but did not undergo apoptosis when induced to express these p53 mutants (data not shown, Table I). However, we found that *p21* was not significantly induced (Fig. 2, *A* and *C*), suggesting that p53-dependent cell cycle arrest in G_1 can be mediated by a gene(s) other than *p21*.

The Proline-rich Domain Contributes to the Ability of p53 to Induce Cell Cycle Arrest—Previously, we and others have shown that the proline-rich domain (16–18) and the C-terminal basic domain (22, 32) are necessary for inducing apoptosis but not cell cycle arrest. To determine whether both domains are dispensable for inducing cell cycle arrest, we generated p53-(ΔPRDΔBD), which lacks both the proline-rich domain and the C-terminal basic domain. We then established several cell lines that inducibly express this mutant. Western blots from three representative cell lines, p53-(ΔPRDΔBD)-2, -6, and -7, are shown in Fig. 2*E*. We found that the level of p53 expressed in p53-(ΔPRDΔBD)-2 cells was comparable with that in p53-3, HA·p53-15, and p53-(ΔBD)-1 but slightly lower than that in p53-(ΔPRD)-5, which inducibly expresses a p53 mutant lacking

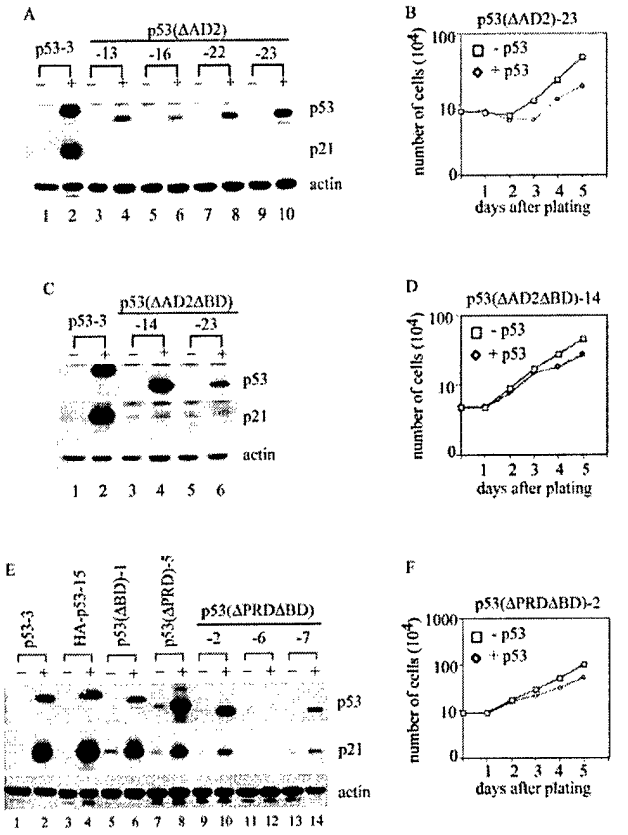

FIG. 2. **The activity for cell cycle arrest but not apoptosis was partially retained in p53-(ΔAD2), p53-(ΔAD2ΔBD), and p53-(ΔPRDΔBD).** *A*, *C*, and *E*, levels of p53, p21, and actin were assayed by Western blot analysis in cell lines as shown in the absence (−) or presence (+) of p53 for 24 h. Antibodies used were as described in the legend to Fig. 1. *B*, *D*, and *F*, growth rates of p53-(ΔAD2)-23, p53-(ΔAD2ΔBD)-14, and p53-(ΔPRDΔBD)-2 cells in the absence (□) or presence (◇) of p53 over a 5-day period.

TABLE I
p53 domain and activity

	Domain					Activity			
	AD1[a]	AD2[a]	PRD[a]	DBD+NLS+TD·NES[a]	BD[a]	p21[b]	arrest[c]	death[d]	Ref
Wild-type						+++	+++	+/−	e
p53(AD1⁻)[a]	XXX					−/+	+/−	++	e
p53(ΔBD)						++	++	−	e
p53(AD1⁻ΔBD)	XXX					−	−	−	e
p53(ΔAD1)						−/+	++	+/−	f
p53(ΔAD1ΔAD2⁻)	XXX					−	−	−	f
p53(ΔAD1ΔAD2)						−	−	−	f
p53(AD1⁻AD2⁻)	XXX					−	−	−	f
p53(ΔPRD)						++	+++	−	g
p53(AD2⁻)[a]		XXX				+/−	+++	+/−	this study
p53(AD2⁻ΔBD)		XXX				+/−	+	−	this study
p53(ΔAD2)						−	+	+/−	this study
p53(ΔAD2ΔBD)						−	+	−	this study
p53(ΔPRDΔBD)						+/−	+	−	this study
p53(ΔAD1ΔBD)						+	+++	+++	this study
p53(ΔAD1AD2⁻ΔBD)	XXX					−	−	−	this study
p53(ΔAD1ΔAD2ΔBD)						−	−	−	this study
p53(ΔAD1ΔPRDΔBD)						−	−	−	this study
p53(ΔAD1ΔPRD)						−	−	−	this study
p53(ΔAD2ΔPRD)						−	−	−	this study
p53(ΔAD2ΔPRDΔBD)						−	−	−	this study

[a] AD1, activation domain 1 within residues 1–42; AD2, activation domain 2 within residues 43–63; PRD, the proline-rich domain within residues 64–92; DBD, the DNA binding domain within residues 100–300; NLS, the nuclear localization signal within residues 316–325; TD, the tetramerization domain within residues 334–356; NES, the nuclear export signal within residues 334–356; BD, the C-terminal basic domain within residues 364–393; AD1⁻, a double point mutation of L22Q and W23S; AD2⁻, a double point mutation of W53Q and F54S.

[b] The ability of p53 to induce p21 was measured by Western and Northern blot analyses.

[c] Arrest was measured by DNA histogram analysis.

[d] Death was measured by trypan blue dye exclusion and annexin V staining assays and DNA histogram analysis.

[e] Ref. 22.

[f] Ref. 10.

[g] Ref. 17.

the proline-rich domain (Fig. 2*E*, *p53 panel*). To determine whether *p21* can be induced, we found that p53-(ΔPRDΔBD) was much less potent in inducing *p21* than wild-type p53, HA-tagged p53, p53-(ΔBD), or p53-(ΔPRD) (Fig. 2*E*, *p21 panel*). However, when the DNA binding activity was determined *in vitro*, we found that p53-(ΔPRDΔBD) was as potent as wild-type p53 in binding to the ribosomal gene cluster p53 response element (data not shown). This suggests that deletion of both the proline-rich domain and the C-terminal basic domain does not affect the activity of the p53 DNA binding domain. Growth rate analysis showed that p53-(ΔPRDΔBD) had a much reduced ability to suppress cell proliferation (Fig. 2*F*). In addition, DNA histogram analysis and annexin V staining assay showed that a partial arrest in G_1, but no apoptosis, was detected in p53-(ΔPRDΔBD)-2 cells (data not shown).

p53-(ΔAD1ΔBD) Is Small but Potent in Inducing Cell Cycle Arrest and Apoptosis—We and others have shown that p53-(ΔBD), which lacks the C-terminal basic domain, is inactive in inducing apoptosis (22, 32, 36) whereas p53-(ΔAD1), which lacks activation domain 1 (residues 1–42), is very active (10). To determine whether the C-terminal basic domain is necessary for p53-(ΔAD1) to induce apoptosis, we generated p53-(ΔAD1ΔBD), which lacks activation domain 1 and the C-terminal basic domain. We then established several cell lines that inducibly express p53-(ΔAD1ΔBD). Western blots from three representative cell lines, p53-(ΔAD1ΔBD)-3, -6, and -7, are shown in Fig. 3*A*. We found that the level of p53 expressed in these cells was comparable with that in p53-3, HA·p53-15, and p53-(ΔBD)-1 cells, but lower than that in p53-(ΔAD1)-2 cells (Fig. 3*A*, *p53 panel*). p53-(ΔAD1)-2 cells are derived from H1299 cells that inducibly express p53-(ΔAD1), which lacks activation domain 1 (10). We found that p53-(ΔAD1ΔBD) retained the ability to induce *p21*. Induction of *p21* by p53-(ΔAD1ΔBD) was greater than induction by p53-(ΔAD1) but less than induction by wild-type p53 and p53-(ΔBD) (Fig. 3*A*, *p21 panel*). Growth rate analysis showed that cells failed to multiply when induced to express p53-(ΔAD1ΔBD) (Fig. 3, *B* and *C*). Microscopic examination showed that the p53-expressing cells detached from plates and shrank to form apoptotic bodies (data not shown). DNA histogram analysis showed that the percentage of cells in S phase decreased from 35 to 11% but the percentage of cells in G_1 increased from 55 to 75%, suggesting that these cells arrested primarily in G_1 (Fig. 3, *D–E*). We also found that the number of cells with a sub-G_1 DNA content was not significantly increased. However, when stained for annexin V, we found that the percentage of stained cells increased from 7 to 31%, suggesting that these cells also underwent apoptosis (Fig. 3, *F–G*).

To further confirm the ability of p53-(ΔAD1ΔBD) to induce apoptosis, we generated several MCF7 breast carcinoma cell lines that inducibly express wild-type p53 and p53-(ΔAD1ΔBD). Western blots from one representative cell line that inducibly expresses wild-type p53 (MCF7-p53-24) and two that inducibly express p53-(ΔAD1ΔBD) (MCF7-p53-

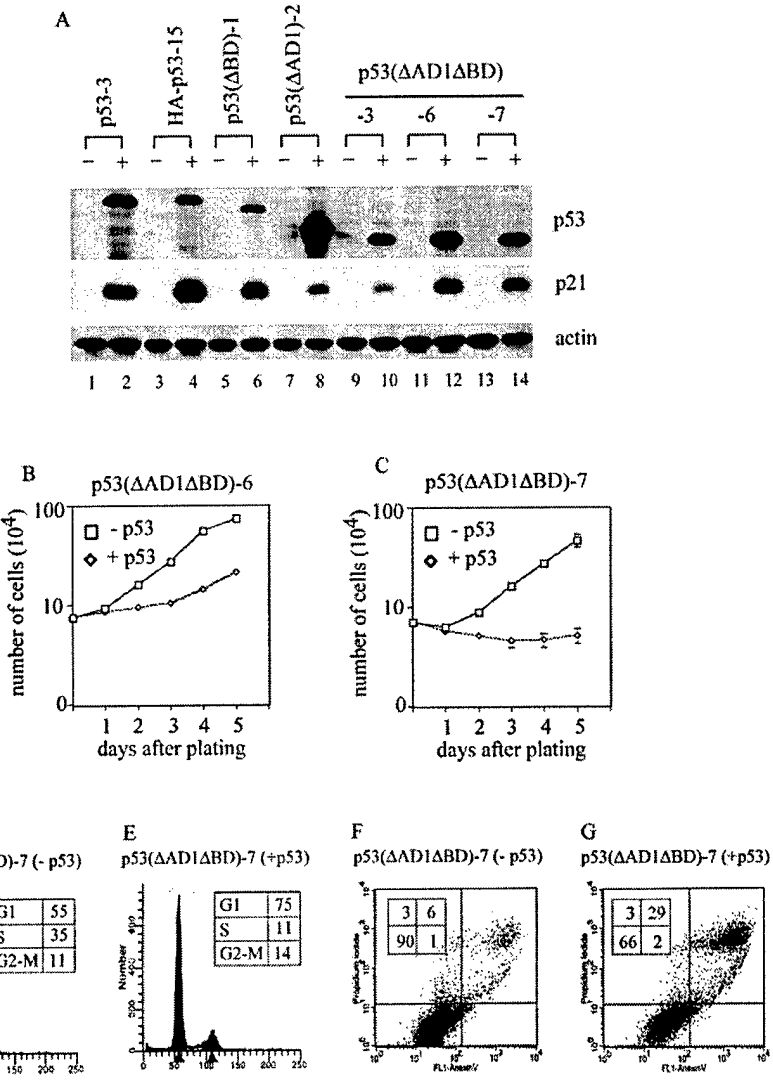

FIG. 3. **The C-terminal basic domain is not necessary for apoptosis when activation domain 1 is absent.** *A*, levels of p53, p21, and actin were assayed by Western blot analysis in cell lines as shown in the absence (−) or presence (+) of p53 for 24 h. Antibodies used were as described in the legend to Fig. 1. *B* and *C*, growth rates of p53-(ΔAD1ΔBD)-6 and p53-(ΔAD1ΔBD)-7 cells in the absence (□) or presence (◇) of p53 over a 5-day period. *D* and *E*, DNA content was quantified by propidium iodide staining of fixed cells that were non-induced (− p53) or induced (+ p53) to express p53-(ΔAD1ΔBD) for 3 days. *F* and *G*, apoptotic cells were quantified by propidium iodide-annexin V staining of cells that were non-induced (− p53) or induced (+ p53) to express p53-(ΔAD1ΔBD) for 3 days.

(ΔAD1ΔBD)-7 and -15) are shown in Fig. 4*A*. We found that the level of p53 induced in MCF7-p53-(ΔAD1ΔBD)-7 and -15 cells was slightly lower than in MCF7-p53-24 cells (Fig. 4*A*, *p53 panel*). When the level of p21 was measured to determine the transcriptional activity of p53-(ΔAD1ΔBD), we found that p53-(ΔAD1ΔBD) was potent in transactivation (Fig. 4*A*, *p21 panel*). This result is similar to that obtained in H1299 cells (Fig. 3*A*). Growth rate analysis showed that cells failed to multiply when induced to express wild-type p53 or p53-(ΔAD1ΔBD) (Fig. 4, *B–C*). Microscopic examination showed that the p53-expressing cells detached from plates and shrank to form apoptotic bodies (data not shown). DNA histogram analysis showed that the percentage of cells that had a sub-G_1 DNA content was increased from 3 to 37% by wild-type p53 (Fig. 4, *D–E*) and from 4 to 49% by p53-(ΔAD1ΔBD) (Fig. 4, *H–I*). In addition, annexin V staining assay showed that the percentage of the annexin V-stained cells was increased from 7 to 28% by wild-type p53 and from 9 to 29% by p53-(ΔAD1ΔBD). These data indicate that p53-(ΔAD1ΔBD) is a potent apoptotic inducer.

At Least Two of the Three Domains, i.e. Activation Domain 1, Activation Domain 2, and the Proline-rich Domain Are Required for Inducing Cell Cycle Arrest—To further define the role of activation domain 1, activation domain 2, the proline-rich domain, and the C-terminal basic domain in inducing cell cycle arrest and apoptosis, we generated six p53 mutants that are dysfunctional in two or three of the four functional domains (Fig. 5). We then established several cell lines that inducibly express these p53 mutants individually (Fig. 5). These are p53-(ΔAD1AD2$^-$ΔBD), Fig. 5*A*; p53-(ΔAD1ΔAD2ΔBD), Fig. 5*B*; p53-(ΔAD1ΔPRDΔBD), Fig. 5*C*; p53-(ΔAD1ΔPRD), Fig. 5*D*; p53-(ΔAD2ΔPRD), Fig. 5*E*; and p53-(ΔAD2ΔPRDΔBD), Fig. 5*E*. The level of p53 expressed in some of these mutant p53-producing cells was comparable with or higher than that in p53-3 cells (Fig. 5, *A–E*, *p53 panel*). However, none of these mutants were capable of inducing *p21* (Fig. 5, *A–E*, *p21 panel*). In addition, cell cycle arrest and apoptosis were not detected by growth rate and DNA histogram analyses and annexin V staining assay (data not shown). These data suggest that at least two of the three domains (activation domain 1, activation domain 2, and the proline-rich domain) are required for p53 activity.

Regulation of p21 and BAX by p53 Mutants—To determine the ability of various p53 mutants that lack activation domain 1, activation domain 2, and/or the C-terminal basic domain in inducing *p21* and *BAX*, we performed a Northern blot analysis (Fig. 6). We found that wild-type p53 was very active (*lanes 1–2*). p53-(R249S), a tumor-derived mutant that is defective in the DNA binding domain, was nearly inert (*lanes 3–4*). Although deletion of the C-terminal basic domain renders p53 constitutively active in binding to DNA *in vitro* (26), the ability of p53-(ΔBD) to induce *p21* and *BAX* was approximately 2-fold less efficient than that of wild-type p53 (compare *lanes 1–2* and

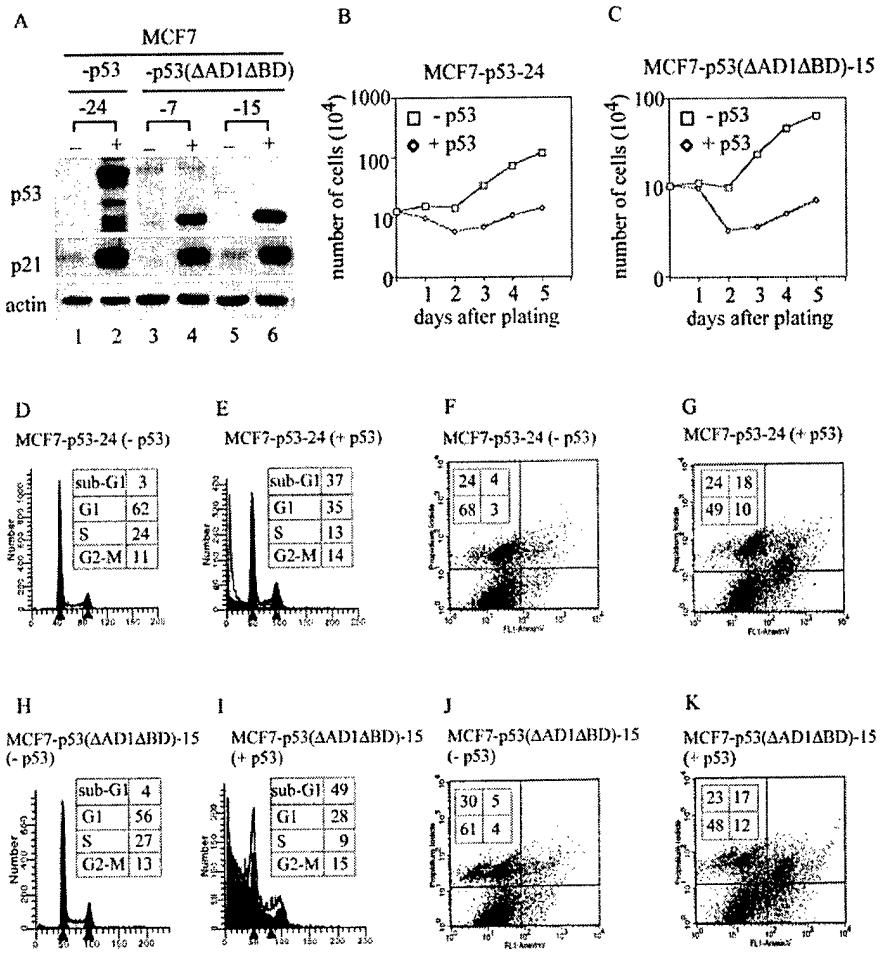

FIG. 4. **p53-(ΔAD1ΔBD) is capable of inducing both cell cycle arrest and apoptosis in MCF7 cells.** *A*, levels of p53, p21, and actin were assayed by Western blot analysis in cell lines as shown in the absence (−) or presence (+) of p53 for 24 h. Antibodies used were as described in the legend to Fig. 1. *B* and *C*, growth rates of MCF7-p53-24 and MCF7-p53-(ΔAD1ΔBD)-15 cells in the absence (□) or presence (◇) of p53 over a 5-day period. *D*, *E*, *H*, and *I*, DNA content was quantified by propidium iodide staining of fixed cells that were non-induced (− p53) or induced (+ p53) for 3 days to express p53 (*D* and *E*) or p53-(ΔAD1ΔBD) (*H* and *I*). *F*, *G*, *J*, and *K*, apoptotic cells were quantified by propidium iodide-annexin V staining of cells that were non-induced (− p53) or induced (+ p53) for 3 days to express p53 (*F* and *G*) or p53-(ΔAD1ΔBD) (*J* and *K*).

7–8). p53-(AD1$^-$) (*lanes 5–6*), p53-(ΔAD1) (*lanes 9–10*), p53-(AD2$^-$) (*lanes 13–14*), and p53-(AD2−ΔBD) (*lanes 15–16*) were extremely weak in inducing *p21* and *BAX* (2-fold or less). It should be mentioned that p53-(AD2$^-$) is extremely potent in inducing G_1 arrest (see Fig. 1, *D–E*), suggesting that a gene(s) other than *p21* is responsible for this. Furthermore, when activation domain 1 and the basic domain were deleted, the ability of p53-(ΔAD1ΔBD) to induce *p21* and *BAX* was partially restored (*lanes 11–12*), consistent with the result detected by Western blot analysis (Fig. 3*A*).

DISCUSSION

p53 induces apoptosis but the underlying mechanism remains unclear. To determine this mechanism, two major questions need to be addressed. What domains in p53 are required and is p53 transcriptional activity necessary for inducing apoptosis? Previous attempts to answer these questions have been inconclusive, because different experimental systems have been used (1, 2). These include various types of cell lines and methods to express p53 (transient *versus* stable, ectopic *versus* inducible) and different types of p53 mutants (temperature-sensitive mutant *versus* wild-type p53; point mutations *versus* deletion mutations). To avoid these problems, we have applied the tetracycline inducible expression system to stably express various p53 mutants in *p53*-null H1299 cells. On the basis of the results obtained in this study (Table I) and several previous studies (11, 12, 16, 32, 36, 38–40), including our own (10, 17, 22), we propose the following model for p53 functional domains in apoptosis (Fig. 7). First, p53 DNA binding activity is necessary for apoptosis because mutants that are defective in the DNA binding and tetramerization domains are inert. Second, activation domain 2 and the proline-rich domain can form an activation domain for transactivating pro-apoptotic genes or transrepressing anti-apoptotic genes, because mutation or deletion in either one of the domains abrogates the apoptotic activity. Third, activation domain 1 is not required because deletion of or mutation in activation domain 1 (p53-(ΔAD1), p53-(AD1$^-$)) has little effect on apoptosis. Fourth, the C-terminal basic domain is necessary for maintaining p53 competent in inducing apoptosis, probably by relieving the inhibitory activity of activation domain 1, because p53-(ΔAD1ΔBD), but not p53-(ΔBD), is capable of inducing apoptosis.

Several p53 inducible genes, such as *BAX* (41), *KILLER/DR5* (42), and several *PIGs* (43), may participate in the apoptotic process. These genes can be induced by either p53-(ΔPRD) (17) or p53-(AD2$^-$) (data not shown), both of which are active in inducing cell cycle arrest but not apoptosis, suggesting that these genes are not required or insufficient for inducing apoptosis. Recent evidence has shown that p53 can repress specific genes, such as MAP4 (44). It is possible that transrepression of anti-apoptotic genes plays an important role in p53-mediated

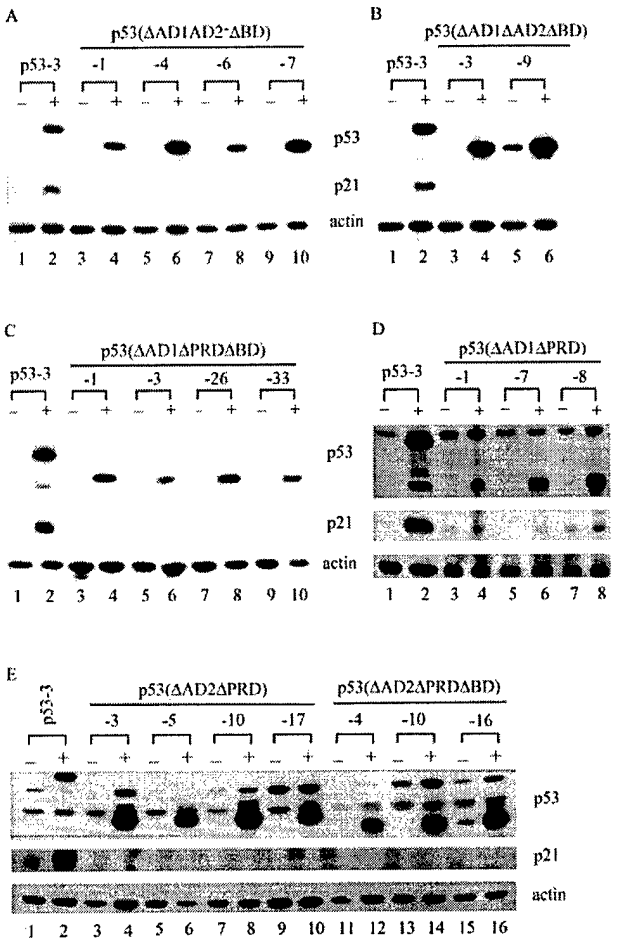

FIG. 5. **At least two of the three domains, *i.e.* activation domain 1, activation domain 2, and the proline-rich domain, are required for inducing cell cycle arrest.** Levels of p53, p21, and actin were assayed by Western blot analysis in cells that were non-induced (−) or induced (+) for 24 h to express p53-(ΔAD1AD2⁻ΔBD) (*A*), p53-(ΔAD1ΔAD2ΔBD) (*B*), p53-(ΔAD1ΔPRDΔBD) (*C*), p53-(ΔAD1ΔPRD) (*D*), p53-(ΔAD2ΔPRD) (*E*, *lanes 3–10*), and p53-(ΔAD2ΔPRDΔBD) (*E*, *lanes 11–16*). Antibodies used were as described in the legend to Fig. 1.

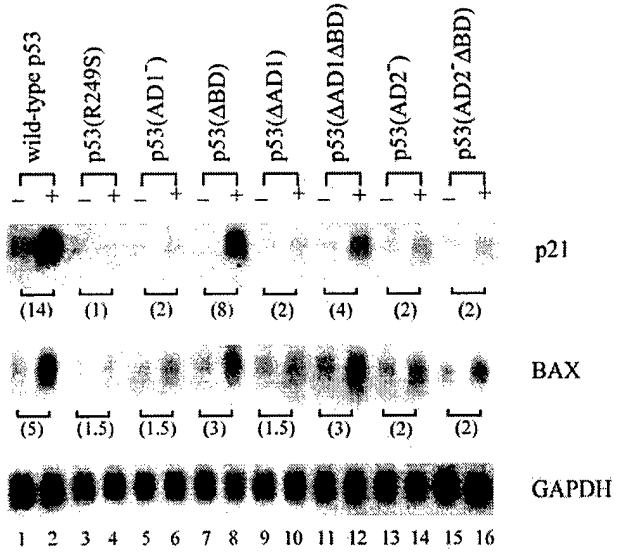

FIG. 6. **Regulation of *p21* and *BAX* by p53 mutants.** A Northern blot was prepared using total RNAs isolated from non-induced cells (−) or cells induced for 24 h to express wild-type p53 or various p53 mutants as shown above the blot (+). The blot was probed with cDNAs derived from the *p21*, *BAX*, and glyceraldehyde-3-phosphate dehydrogenase genes, respectively. After normalization to the amount of glyceraldehyde-3-phosphate dehydrogenase transcripts, the levels of induction by wild-type p53 or various p53 mutants were quantified by phosphorimager and are shown below the blot.

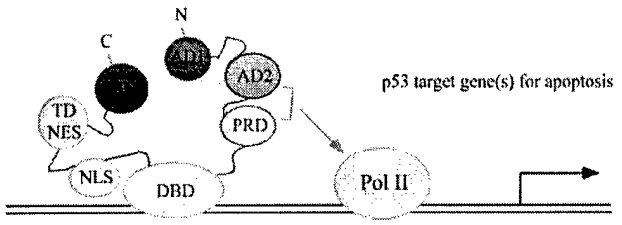

FIG. 7. **A model of apoptosis for p53 functional domains.**

apoptosis. Therefore, the cell lines that inducibly express the p53 mutants described in this study, especially p53-(ΔAD1ΔBD), can be used to identify and determine whether a cellular gene is necessary for mediating p53-dependent apoptosis.

p53 transcriptional activity has been shown to be necessary for inducing cell cycle arrest (1, 2, 4, 45). In this study, we extend this observation. We found that an activation domain capable of inducing at least partial cell cycle arrest can be formed by activation domain 1 plus activation domain 2, activation domain 1 plus the proline-rich domain, or activation domain 2 plus the proline-rich domain (Table I). When two of the three domains, *i.e.* activation domain 1, activation domain 2, and the proline-rich domain, become dysfunctional, the activity in cell cycle arrest is abrogated (Table I). It should be mentioned that p53-(AD1⁻) is defective in inducing cell cycle arrest although two functional domains, *i.e.* activation domain 2 and the proline-rich domain are still intact (22). However, when part or all of the residues for activation domain 1 are deleted, as in p53-(Δ1–23) and p53-(ΔAD1), the ability to induce cell cycle arrest is retained. This suggests that the presence of the mutated activation domain 1 may mask the activity of, or inhibit the interaction of, a potential co-activator (or an adaptor) with the activation domain formed by activation domain 2 and the proline-rich domain necessary for transactivation or transrepression.

The search for mediators of p53-dependent cell cycle arrest has identified many cellular p53 target genes (1, 4, 46). $p21^{cip1/waf1}$, a well characterized cyclin-dependent kinase inhibitor, can mediate cell cycle arrest in G_1 when overexpressed (22, 47–51). Previous studies have shown that p53-(AD1⁻), which is deficient in inducing *p21*, is incapable of inducing arrest in G_1, consistent with the hypothesis that p21 plays an important role in mediating p53-dependent arrest in G_1 (22, 40). In this study, we found that p53-(AD2⁻) is extremely active in inducing arrest in G_1, suggesting that activation domain 1, but not activation domain 2, plays an important role in inducing cell cycle arrest. However, *p21* is only slightly induced by p53-(AD2⁻) (Fig. 1*A*). Because p53-(AD1⁻AD2⁻), which is deficient in both activation domain 1 and activation domain 2, is inert in inducing cell cycle arrest (9–11), this suggests that a gene(s) responsible for arrest by p53-(AD2⁻) must be induced. This is not surprising because DNA damage-induced G_1 arrest is delayed but not abolished in *p21*-null fibroblasts from p21-deficient mice (52, 53). Therefore, the cell line that inducibly expresses p53-(AD2⁻) can be used to identify other novel gene(s) responsible for G_1 arrest.

Previously, several studies have shown that the p53 protein can be cleaved by cellular proteases in cells treated with DNA damaging agents, which leads to formation of several smaller polypeptides with molecular masses ranging from 35–50 kDa (54–58). In addition, the cleavage of p53 is concomitant with the

onset of apoptosis in cells treated with DNA damaging agents, suggesting that the cleaved p53 polypeptides are potent in p53 activity and may participate in the apoptotic process (58). Interestingly, one of the cleaved p53 polypeptides, p50, is p53-(ΔN23), which lacks the N-terminal 23 residues (58). We have shown previously that p53-(ΔN23) is active in inducing cell cycle arrest and apoptosis (10). Thus, the cellular machinery can generate an active but smaller p53 polypeptide that would not be subject to negative regulation by MDM2 (59–63). It is not clear whether p53-(ΔAD1ΔBD) is an *in vivo* cleavage product of p53. However, because p53-(ΔAD1ΔBD) lacks the MDM2 binding site, it would not be subjected to the negative regulation by MDM2. Thus, p53-(ΔAD1ΔBD) represents a small but potent, apoptosis-inducing form of p53. Recent clinical trials have shown that adenoviruses expressing p53 are effective in treating some advanced forms of human cancers (64, 65). We suggest that p53-(ΔAD1ΔBD) is a good candidate to replace the larger, unwieldy wild-type p53 in cancer gene therapy.

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Appendix 5

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SHORT REPORT

p73 is transcriptionally regulated by DNA damage, p53, and p73

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p73 is a member of the p53 family. Recent studies have shown that DNA damage can stabilize p73 protein and enhance p73-mediated apoptosis in a c-Abl dependent manner. To determine what regulates p73 transcriptionally, we analysed the expression of p73 in several cell lines following genotoxic stresses. We found that p73 is induced in certain cell lines when treated with therapeutic DNA damaging agents. We also found that p53 and p73, but not mutant p53(R249S) and p73β292, directly induce the expression of the p73 gene. In addition, we found one potential p53-binding site in the promoter of the p73 gene. This binding site is responsive to p53, p73, and DNA damage. Taken together, these data suggest that p73 is transcriptionally regulated by DNA damage and p53, and is autoregulated. *Oncogene* (2001) **20,** 769–774.

Keywords: p53; p73; DNA damage; transcriptional regulation

p73 was identified as a member of the p53 family since the residues in p73 and p53 are highly similar, especially in the central sequence-specific DNA binding domain, the amino terminal activation domain, and the carboxyl terminal oligomerization domain (Kaghad *et al.*, 1997). p73 is expressed in at least six alternatively spliced forms, that is, p73α, p73β, p73γ, p73δ, p73ε, and p73ζ (De Laurenzi *et al.*, 1998, 1999; Kaghad *et al.*, 1997; Zaika *et al.*, 1999). Like p53, p73 can induce cell cycle arrest and apoptosis when overexpressed in cells (Jost *et al.*, 1997; Kaghad *et al.*, 1997; Zhu *et al.*, 1998a). As a sequence-specific transcription factor, p73 can recognize several p53 response elements both *in vitro* and *in vivo* (Chen, 1999; Kaelin, 1999). Loss of p73 transcriptional activity abrogates its ability to induce cell cycle arrest and apoptosis. Despite these similarities to p53, p73 regulates some cellular p53 target genes differently from p53 (Di Como *et al.*, 1999; Yu *et al.*, 1999; Zhu *et al.*, 1998a). For example, 14-3-3σ, which may mediate p53-dependent G_2-M arrest, is activated several fold higher by p73 than by p53. Furthermore, unlike mice lacking p53 (Donehower *et al.*, 1992), p73 deficient mice are not susceptible to spontaneous tumors, but instead develop neurological, pheromonal, and inflammatory defects (Yang *et al.*, 2000). These results suggest that the signaling pathways for p53 and p73 may be similar but also have important differences. Recent studies have shown that p73 can be stabilized and phosphorylated at a tyrosine residue by DNA damage in a c-Abl-dependent manner, leading to an enhanced p73-mediated apoptotic response (Agami *et al.*, 1999; Gong *et al.*, 1999; Yuan *et al.*, 1999). In this study, we found that p73 is transcriptionally regulated by DNA damage, p53 and p73.

To determine whether p73 can be induced transcriptionally by DNA damage, we analysed the expression of the p73 gene by Northern blot analysis in LS174T, T98G, and SW480 cells following genotoxic stresses. LS174T and SW480 are colorectal carcinoma cell lines, and T98G is a glioma cell line. These cells were treated with four therapeutic agents, camptothecin, etoposide, cisplatin, or doxorubicin, to induce DNA damage. We found that p73 was significantly induced in LS174T, SW480 and T98G cells when treated with camptothecin (Figure 1a), etoposide, cisplatin, or doxorubicin (data not shown). We also tested the expression of p21, a well-defined target of p53 (el-Deiry *et al.*, 1993). We found that p21 was significantly induced only in LS174T, but not in T98G and SW480, cells. This is consistent with previous reports that in LS174T cells, the endogenous wild-type p53 protein is stabilized and capable of inducing p21 (Zhu *et al.*, 1999), whereas in T98G and SW480 cells, mutant p53 may antagonize the activity of p73 to induce p21 (Di Como *et al.*, 1999; Marin *et al.*, 2000). To determine whether DNA damage induction of p73 correlates with an increased expression of p73, the level of p73 protein was quantified in cells that were untreated or treated with camptothecin. We found that p73 protein was undetectable when cell extracts were used directly for Western blot analysis. This is probably due to the low affinity of the anti-p73 antibody or the low abundance of p73 protein expressed in cells. To circumvent this problem, we used mouse anti-p73 monoclonal antibody to immunoprecipitate p73 and the resulting precipitates were used to detect p73 by Western blot analysis with rabbit anti-p73 polyclonal antibody. We found that the level of both p73α and p73β proteins was significantly increased (>3 fold) in SW480 and T98G cells (Figure 1b) and in LS174T cells (Figure 1c).

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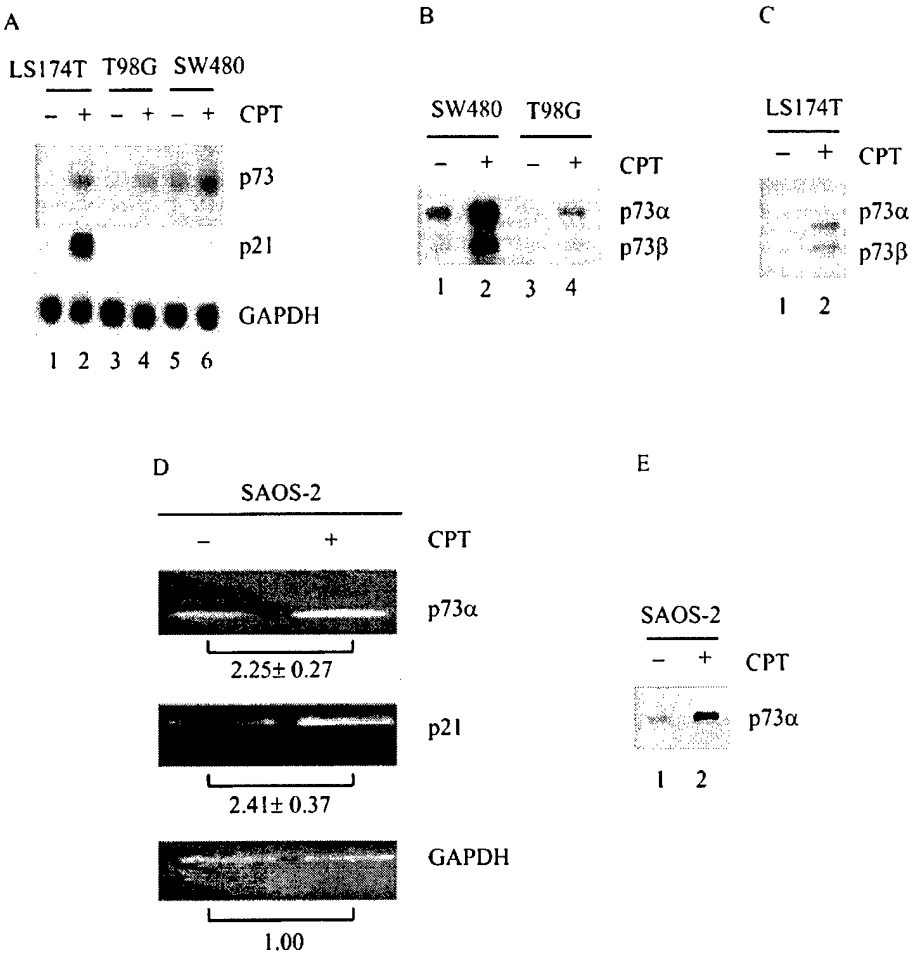

Figure 1 Upregulation of p73 by DNA damage. (**a**) The expression of the p73 gene is induced by DNA damage in LS174T, T98G, and SW480 cells. A Northern blot was prepared using 40 μg of total RNA isolated from LS174T, T98G, and SW480 cells that were untreated (−) or treated (+) with 300 nM camptothecin (CPT) for 24 h. Total RNA was isolated using Trizol reagents (Life Technologies, Inc., Gaithersburg, MD, USA). Northern blot analysis was performed as described (Zhu *et al*., 1998b). The probes to detect p21 and GAPDH were prepared as previously described (Zhu *et al*., 1998b). The probe to detect human p73 was amplified by PCR with the following primers: forward primer (5′-AAG ATG GCC CAG TCC ACC GCC-3′), and reverse primer (5′-GCG GAT CCT CAG GGC CCC CAG GTC CT-3′). The amplified p73 cDNA was cloned and confirmed by sequencing to be derived from the p73 gene. The blot was probed with p73 cDNA, and then reprobed with both p21 and GAPDH cDNAs. (**b** and **c**) The level of p73 protein is increased by DNA damage in SW480, T98G and LS174T cells. Cell extracts were prepared from SW480, T98G and SW480 cells, which were untreated (−) or treated (+) with 300 nM camptothecin (CPT) for 24 h. An equal amount of cell extracts from both the control and treated cells was used for immunoprecipitation with mouse anti-p73 monoclonal antibody (Ab-2; Oncogene Research Products, Cambridge, MA, USA). The amount of p73 protein in the precipitates was quantified by Western blot analysis with rabbit anti-p73 polyclonal antibody (Ab-4; Oncogene Research Products). (**d**) The expression of the p73 gene is induced by DNA damage in SAOS-2 cells. Total RNA was purified from SAOS-2 cells that were untreated or treated with 300 nM camptothecin (CPT) for 24 h. First-strand cDNA was synthesized using Superscript reverse transcriptase (Life Technologies, Inc.) according to the manufacturer's instruction. The levels of the transcripts for p73α, p21, and GAPDH were determined by PCR with 35, 30 and 25 cycles, respectively. The primers used to amplify a 405-bp p73α cDNA fragment are forward primer (5′-TTT AAC AGG ATT GGG GTG TC-3′) located in p73 exon 13, and reverse primer (5′-CGT GAA CTC CTC CTT GAT GG-3′) located in p73 exon 14. The primers used to amplify p21 were forward primer (5′-AGG CAC CGA GGC ACT CAG AG-3′) and reverse primer (5′-AAG CCG GCC CAC CCA ACC TC-3′). The primers used to amplify GADPH are forward primer (5′-TGA AGG TCG GAG TCA ACG GAT TTG GT-3′), and reverse primer (5′-CAT GTG GGC CAT GAG GTC CCC AC-3′). (**e**) The level of p73 protein is increased by the DNA damage agent camptothecin (CPT) in SAOS-2 cells. The experiment was performed similarly as in (**b**) except that anti-p73α antibody (Ab1: Oncogene Research Products) was used for immunoprecipitation

Next, we determined DNA damage induction of p73 in p53-null SAOS-2 cells. We used RT–PCR with a pair of primers that specifically amplify the endogenous p73α transcript in SAOS-2 cells that were untreated or treated with camptothecin. We found that p73α was induced (Figure 1d, p73 panel). As a positive control, we found that p21 was also induced (Figure 1d, p21 panel). In addition, DNA damage induction of p73 correlated with an increased expression of p73 protein (Figure 1e).

Since the p53 pathway is functional in LS174T cells, we wanted to determine whether DNA damage induction of p73 is mediated by p53. To do this, we

analysed the expression of p73 in p53-7 and p53(R249S)-4 cell lines, both of which are derivatives of SAOS-2 cells that express wild-type p53 and mutant p53 (R249S), respectively, under a tetracycline-regulated promoter (Figure 2a). We found that p73 was induced in SAOS-2 cells by wild-type p53, but not by mutant p53(R249S) (Figure 2b). We also found that like p21, the level of p73 protein was increased by p53 (Figure 2c). These results indicate that p73 is a potential p53 target.

Previously, we and others have shown that p73 can differentially regulate some p53 target genes (Di Como *et al*., 1999; Yu *et al*., 1999; Zhu *et al*., 1998a). Thus, we tested whether p73 is also capable of regulating itself. To do this, SAOS-2 cells were transfected with a pcDNA3 control vector or a pcDNA3 vector that expresses p73β or p73β292. RT–PCR was performed to specifically amplify the endogenous p73α transcript. We found that like induction of p21 (twofold), endogenous p73α was induced by exogenous wild-type

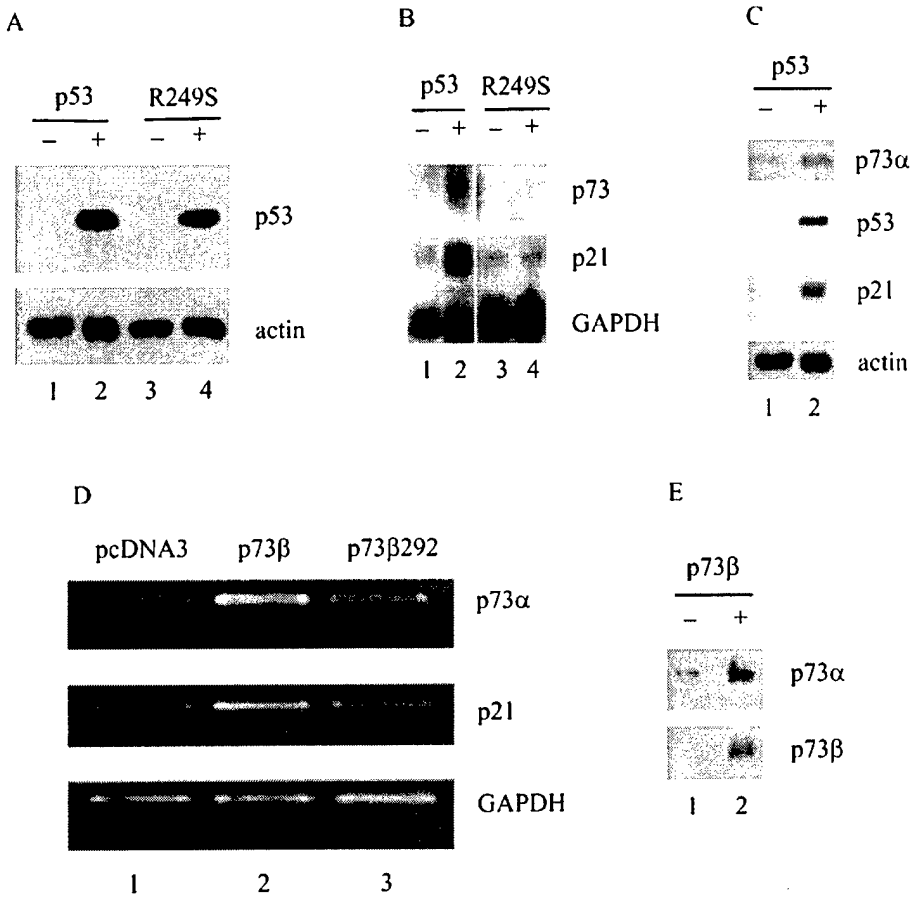

Figure 2 Upregulation of p73 by p53 and p73. (**a**) An equivalent level of the wild-type p53 and mutant p53(R249S) proteins was expressed in p53-7 and p53(R249S)-4 cell lines. Cell extracts were prepared from p53-7 and p53(R249S)-4 cells, that were uninduced (−) or induced (+) to express wild-type p53 and mutant p53(R249S), respectively. The levels of p53 and actin were quantified by Western blot analysis with anti-p53 antibody (PAb240) and anti-actin antibody (Sigma Chemical Co., St. Louis, MO, USA), respectively. (**b**) Wild-type p53, but not p53 mutant, induces p73 in SAOS-2 cells. Northern blots were prepared using 40 μg of total RNA isolated from p53-7 or p53(R249S)-4 cells that were uninduced (−) or induced (+) to express wild-type p53 and mutant p53(R249S), respectively. The experiment was performed similarly as in Figure 1a. (**c**) The level of p73 protein is increased by p53 in SAOS-2 cells. Cell extracts were prepared from p53-7 cells that were uninduced (−) or induced (+) to express p53. An equal amount of cell extracts from both the control and treated cells was used for immunoprecipitation with mouse anti-p73α monoclonal antibody (Ab-1). The level of p73 protein in the precipitates was quantified by Western blot analysis with rabbit anti-p73 polyclonal antibody (Ab-4). The levels of p21, p53, and actin proteins were quantified directly by Western blot analysis with anti-p21 antibody (Ab-1; Oncogene Research Products, Cambridge, MA, USA), anti-p53 antibody (PAb240), anti-actin antibody, respectively. (**d**) The expression of the p73 gene is induced by wild-type p73β, but not by mutant p73β292, in SAOS-2 cells. SAOS-2 cells were transiently transfected with a pcDNA3 control vector, or a pcDNA3 vector that expresses wild-type p73β or mutant p73β292. Total RNA was purified 48 h after transfection. RT–PCR was used to quantify the level of endogenous p73α, p21, and GAPDH transcripts. The experiment was performed similarly as in Figure 1d. (**e**) The level of endogenous p73α protein is increased by exogenous p73β in SAOS-2 cells. SAOS-2 cells were transiently transfected with a pcDNA3 control vector, or a pcDNA3 vector that expresses flag-tagged p73β. Cell extracts were prepared 48 h after transfection and immunoprecipitated with anti-p73α antibody (Ab-1) or anti-flag epitope antibody (Sigma Chemical). The levels of endogenous p73α and exogenous p73β proteins were quantified by Western blot analysis with anti-p73α antibody (Ab-1) and anti-flag epitope antibody, respectively

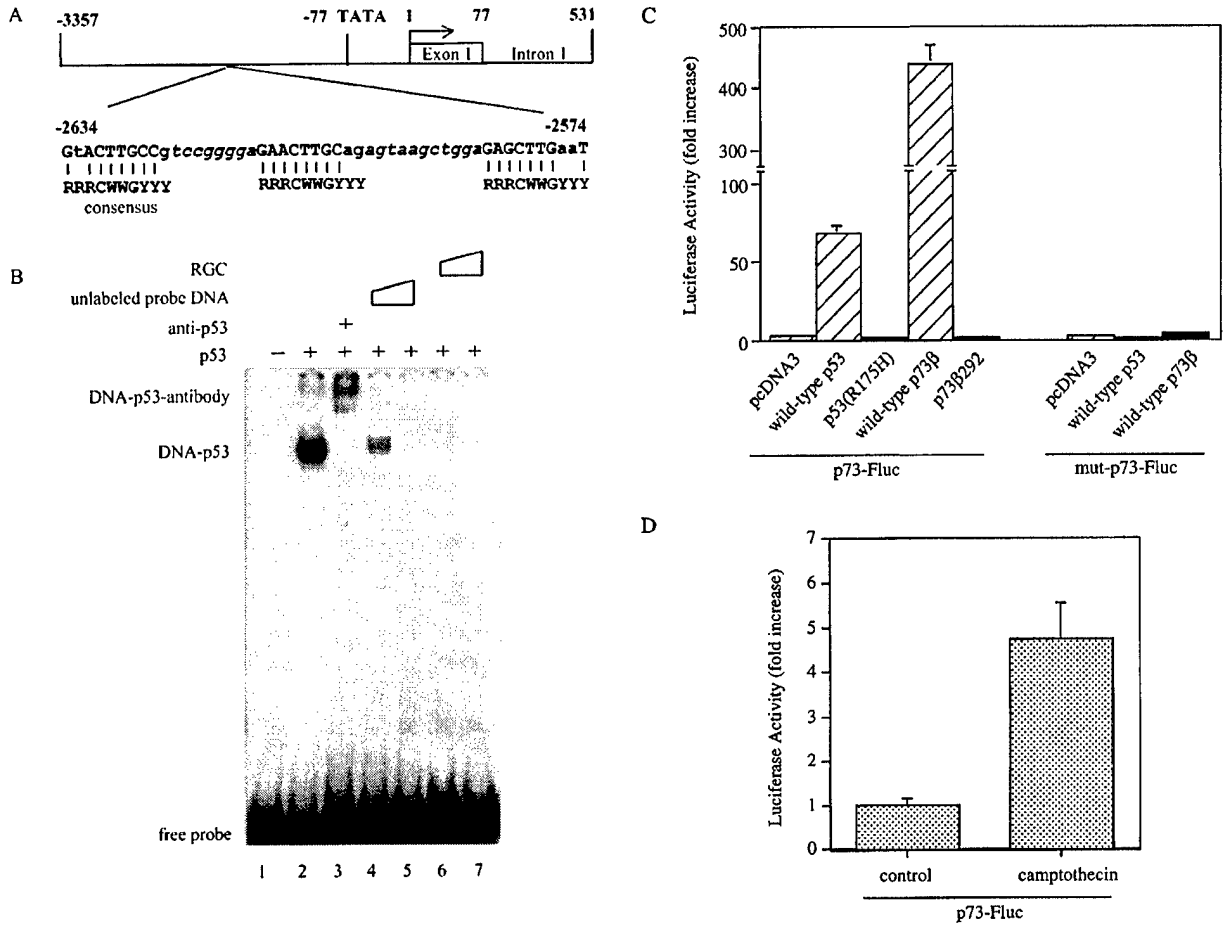

Figure 3 Identification of a p53 response element in the p73 gene. (**a**) Schematic representation of the p73 genomic DNA structure. The positions of the potential p73 transcription start site and a potential p53 response element are indicated. Shown below the genomic structure is the sequence of the potential three half p53 binding sites and the previously characterized p53 consensus response element (el-Deiry *et al*., 1992). R represents purine, Y pyrimidine, and W adenine or thymidine. (**b**) p53 binds specifically to the potential p53 response element *in vitro*. A 61-bp oligonucleotide fragment containing the potential p53 response element in the p73 gene (GGA TCC GTA CTT GCC GTC CGG GGA GAA CTT GCA GAG TAA GCT GGA GAG CTT GAA TGG ATC C) was labeled with α-^{32}P-dCTP. 5 ng of the labeled probe DNA was added to a mixture [20 mM HEPES (pH 7.9), 25 mM KCl, 0.1 mM EDTA, 10% glycerol, 2 mM $MgCl_2$, 2 mM spermidine, 0.5 mM DTT, 0.025% NP-40, 100 ng double-stranded poly(dI:dC), and 2 μg BSA] containing 20 ng of p53 protein. The p53 protein was expressed in a baculovirus expression system and affinity-purified using anti-p53 monoclonal antibody Pab421. The p53-DNA complex was resolved in a 4% polyacrylamide gel. For 'supershifting' the p53-DNA complex, 1 μg of anti-p53 monoclonal antibody Pab1801 was added in the reaction in lane 3. For competition assays, unlabeled probe DNA and RGC (20 and 100 ng) were added to the reaction run in lanes 4,5 and 6,7, respectively. (**c**) The potential p53-binding site, but not its mutated version, in the p73 gene is responsive to wild-type p53 and p73. To generate the luciferase reporter vector, the 61-bp fragment used in (**b**) was cloned upstream of a minimal c-*fos* promoter and a firefly luciferase reporter gene (Johansen and Prywes, 1994). The resulting construct was designated as p73-Fluc. A mutant version of the potential three half p53 binding sites was made and similarly cloned. The resulting construct was designated as mut-p73-Fluc. We co-transfected 2 μg of p73-Fluc or mut-p73-Fluc into H1299 cells with 1 μg of pcDNA3 control vector or a vector that expresses p53, p53(R175H), p73β, or p73β292, and 55 ng of renilla luciferase assay vector, pRL-CMV (Promega, Madison, WI, USA), was also co-transfected as an internal control. Dual luciferase assay was performed according to the manufacturer's instruction (Promega). The fold increase in relative luciferase activity is a product of the luciferase activity induced by p53 or p73 divided by that induced by pcDNA3. (**d**) The potential p53-binding site in the p73 gene is responsive to DNA damage. p73-Fluc was transfected into SAOS-2 cells, which were split into two groups 24 h following transfection. One group was then mock treated whereas the other was treated with 300 nM camptothecin for 24 h. The experiment was performed similarly as in (**c**)

p73β (1.9-fold), but not by mutant p73β292 (Figure 2d). In addition, p73β induction of p73α correlated with an increased expression of endogenous p73α protein (Figure 2e). It should be noted that the magnitude of the p73β effect on the induction of p73α may be underestimated due to untransfected cells in these transient transfection assays. Therefore, p73 can be autoregulated in a manner similar to that of p53 (Deffie *et al*., 1993).

To extend the above observations that p73 is a potential target of p53 and of itself, we searched for a potential p53 response element by sequencing approximately 3.4-kb of genomic DNA in the promoter region of the p73 gene. We found three potential half-binding

sites for p53 located at approximately 2.6-kb upstream of the p73 transcription start site (Figure 3a). When aligned with the consensus p53 binding site (el-Deiry *et al.*, 1992), each of the three half sites (GtACTTGCCg tccgggga GAACTTGCag agtaagctgga GAGCTTGaaT) has two mismatches (underlined lower case letters) in non-critical positions (Figure 3a).

To analyse whether p53 binds to the potential p53 response element, a 61-bp DNA fragment containing this element was synthesized, ^{32}P-labeled, and used in an electrophoretic mobility shift assay (EMSA). We found that when the purified p53 protein was mixed with the DNA fragment, a complex that presumably contained both p53 and DNA was detected (Figure 3b, lane 2). The complex was 'supershifted' with the anti-p53 monoclonal antibody Pab1801 (Figure 3b, lane 3). We also used unlabeled probe DNA and a DNA fragment from the ribosomal gene cluster (RGC) that contains a p53-binding site (Kern *et al.*, 1991) as competitors. We found that both the unlabeled probe DNA and the unlabeled RGC competed with the ^{32}P-labeled probe DNA and inhibited the formation of the p53-DNA complex in a dose-dependent manner (lanes 4–7). These results indicate that p53 interacts specifically with the potential p53 response element in the p73 gene.

To examine whether the potential p53-binding site is responsive to p53 and p73, the 61-bp fragment used in Figure 3b was cloned upstream of a minimal c-*fos* promoter and a luciferase reporter gene (Johansen and Prywes, 1994) to generate a reporter vector designated p73-Fluc. p73-Fluc was co-transfected into H1299 cells with either pcDNA3 control vector or a vector that expresses p53, p53(R175H), p73β or p73β292. We found that the luciferase activity for p73-Fluc was markedly increased by wild-type p53 and p73β, but not by mutant p53(R175H) and p73β292 (Figure 3c). These results are consistent with the above observations that wild-type p53 and p73β, but not mutant p53(R249S) and p73β292, induces p73 (Figure 2). We also substituted six nucleotides in the potential p53 response element predicted to be critical for p53-binding (shown in lower case) (GTAaTTtCCG TCCGGGGA GAAaTTtCAG AGTAAGCTGGA GAGaTTtAATC). We then generated a reporter vector designated mut-p73-Fluc. Mut-p73-Fluc was co-transfected into H1299 cells with either pcDNA3 control vector or a vector that expresses either wild-type p53 or p73β. We found that the luciferase activity for mut-p73-Fluc was not substantially increased by wild-type p53 and p73 (Figure 3c). To determine whether the DNA damage induction of the p73 transcript is due to transcriptional activation or mRNA stabilization, we measured the luciferase activity for p73-Fluc in SAOS-2 cells in the presence or absence of DNA damage. We found that the luciferase activity was substantially increased by DNA damage (Figure 3d), suggesting that DNA damage can activate p73 expression transcriptionally.

In this study, we found that DNA damage, p53 and p73 can transcriptionally activate p73. Since both p53 and p73 proteins can be stabilized by DNA damage, we propose that in normal cells, DNA damage stabilizes and activates p53 and p73, and the resulting activated p53 and p73 proteins can each induce the expression of cellular target genes, including the p73 gene itself. It should be mentioned that p73 is induced by DNA damage or by p53 in SAOS-2, LS174T, SW480, and T98G cells (Figures 1 and 2), but not in other cells, such as H1299 and HCT116 (data not shown). It is still unclear why p73 is not induced in these cells. However, several possibilities exist. First, in some tissue and cell lines, p73 is expressed from only one allele due to genomic imprinting (Kaghad *et al.*, 1997). Thus, a hemizygous deletion of the expressed allele would result in total loss of p73 expression. Second, since DNA damage stabilization of p73 requires the c-Abl pathway (Agami *et al.*, 1999; Gong *et al.*, 1999; Yuan *et al.*, 1999), failure to induce p73 by DNA damage in some cell lines, for example, HCT116, may be due to defects in the c-Abl pathway (Gong *et al.*, 1999). Finally, it is also possible that p73 would not be induced when cells are defective in an additional co-activator that is required for induction of p73. Therefore, future studies are needed to address these questions.

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Appendix 6

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MCG10, a Novel p53 Target Gene That Encodes a KH Domain RNA-Binding Protein, Is Capable of Inducing Apoptosis and Cell Cycle Arrest in G_2-M

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p53, a tumor suppressor, inhibits cell proliferation by inducing cellular genes involved in the regulation of the cell cycle. *MCG10*, a novel cellular p53 target gene, was identified in a cDNA subtraction assay with mRNA isolated from a p53-producing cell line. *MCG10* can be induced by wild-type but not mutant p53 and by DNA damage via two potential p53-responsive elements in the promoter of the *MCG10* gene. The *MCG10* gene contains 10 exons and is located at chromosome 3p21, a region highly susceptible to aberrant chromosomal rearrangements and deletions in human neoplasia. The *MCG10* gene locus encodes at least two alternatively spliced transcripts, MCG10 and MCG10as. The MCG10 and MCG10as proteins contain two domains homologous to the heterogeneous nuclear ribonucleoprotein K homology (KH) domain. By generating cell lines that inducibly express either wild-type or mutated forms of MCG10 and MCG10as, we found that MCG10 and MCG10as can suppress cell proliferation by inducing apoptosis and cell cycle arrest in G_2-M. In addition, we found that MCG10 and MCG10as, through their KH domains, can bind poly(C) and that their RNA-binding activity is necessary for inducing apoptosis and cell cycle arrest. Furthermore, we found that the level of the poly(C) binding MCG10 protein is increased in cells treated with the DNA-damaging agent camptothecin in a p53-dependent manner. These results suggest that the MCG10 RNA-binding protein is a potential mediator of p53 tumor suppression.

RNA-binding proteins are a large family of proteins with diverse functions which contain one or more RNA-binding domains (RBDs) and other auxiliary domains for protein-protein interaction and subcellular targeting (22, 23, 46, 65, 71, 78). Several ribosomal proteins are RNA-binding proteins, for example, S6, S15, and L11, which are necessary for ribosome assembly and may be a target for translational regulation (23, 82). Several groups of RNA-binding proteins have been shown to play an important role in alternate splicing, RNA editing, and alternate poly(A) site selection. Among these are the abundant heterogeneous nuclear ribonucleoproteins (hnRNPs), which shuttle between the nucleus and cytoplasm (48, 74, 82).

Three major RNA-binding motifs have been found in hnRNPs, that is, the RBD, arginine/glycine-rich box (RGG), and hnRNP K homology (KH) domain. The consensus RBD structure is composed of 90 to 100 amino acids with a βαββαβ secondary structure (9). A majority of hnRNPs, such as A, B, C, D, F, G, and H, contain one or more RBDs, which are necessary for the ability of these hnRNPs in the regulation of splicing, RNA trafficking, and mRNA stability (48, 82). RNA-binding experiments demonstrate that RBD motif proteins can bind RNA with a wide range of affinities and specificities (9). The RGG box is composed of several closely spaced arginine-glycine-glycine repeats with a β-spiral secondary structure (9). Several hnRNPs contain RGG boxes along with RBD or KH motifs. RNA-binding experiments have demonstrated that the RGG box has a relatively weak RNA-binding affinity and specificity (9, 48, 82). However, the RGG box can unstack RNA bases and destabilize RNA secondary structures, which enhances RNA binding for one or more other RNA-binding motifs present in the protein. The KH domain consists of 50 to 70 amino acids with a stable βααββα secondary structure (9, 48, 66, 74, 82). A potential surface for RNA binding is centered on the loop between the first two helices (66). The KH motif proteins have a relatively high binding affinity for dCdT elements and cytosine-rich RNA elements, such as oligo(C) polymer and CU-rich elements (74). Several hnRNPs contain one or more KH domains, for example, hnRNP K and E. The KH motif hnRNPs have been shown to play a role in the regulation of transcriptional activation and repression, mRNA stability, and translational silencing (48, 74, 82). Sam68, a target of the Src tyrosine kinase in mitosis, contains one KH domain (4, 53). Interestingly, a splicing variant, Sam68ΔKH, which lacks the KH domain inhibits cell proliferation and cell cycle transition from G_1 to S (4). The fragile X syndrome gene *FMR1* encodes an RNA-binding protein with two KH domains (83). Transcriptional silencing of *FMR1* or a mutation in the C-terminal KH domain leads to fragile X syndrome (93, 96).

p53, a cellular gatekeeper, plays an important role in the regulation of numerous processes, including cell cycle progression and apoptosis (1, 13, 34, 46, 52), differentiation (2), senescence (52), and tumor surveillance (110). Many studies have shown that p53 transcriptional activity is required to regulate these processes (3, 76, 92, 108, 109). Consistent with this idea, the majority of tumor-derived mutations in p53, which is the most frequently mutated gene in human cancers, occurs in the central, conserved sequence-specific DNA-binding domain, which is necessary for transactivation (34, 46). A number of cellular genes have been found to be induced by p53 (27, 46). They can be classified into three major functional groups: (i) genes whose products are capable of mediating p53-dependent cell cycle arrest (13, 27, 46), (ii) genes whose products are capable of mediating p53-dependent apoptosis (13, 27, 46), and (iii) genes whose products are capable of mediating other p53 activities, such as TAP1, which is involved in tumor sur-

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veillance (110), the p48 xeroderma pigmentosa gene which is involved in nucleotide excision repair (37), and the KAI1 gene, involved in suppression of metastasis (56).

Several cellular genes are capable of mediating p53-dependent cell cycle arrest. p21, a well-characterized inhibitor of cyclin-dependent kinase, can induce arrest in G_1 (1, 46, 52) and can also induce G_2-M arrest in cells that harbor a dysfunctional retinoblastoma (RB) gene (69). G_2-M arrest can be induced by 14-3-3σ (12, 35), which inhibits Cdc25C phosphatase activity; GADD45 (95), which is necessary for maintaining genome stability and DNA repair; B99 (88), which is a microtubule-localized protein with G_2-phase-specific expression; and B-cell translocation gene 2 (*BTG2*) (79), whose loss disrupts G_2-M arrest when cells are treated with DNA-damaging agents.

Several candidate genes may mediate p53-dependent apoptosis. These are *bax* (62), *KILLER/DR5* (103), phosphatidyl inositol 3-kinase regulatory subunit p85 (105), *PAG608* (39, 90), *Siah-1* (57, 78), cathepsin D (104), and CD95 (also called Apo-1 or Fas) receptor (5, 64). Nevertheless, it is still not clear whether these p53 targets are necessary or sufficient for inducing apoptosis. Since p53 transcriptional activity is necessary for inducing apoptosis, it is likely that one or more cellular genes must be involved in mediating p53-dependent apoptosis.

In the search for novel cellular target genes responsible for p53 tumor suppression, we performed a cDNA subtraction assay and found one gene, *MCG10*, that is specifically induced by wild-type but not mutant p53 and by DNA damage. This induction occurs via two potential p53-responsive elements. The *MCG10* gene, located at chromosome 3p21 with 10 exons, encodes at least two alternatively spliced transcripts, MCG10 and MCG10as. The MCG10 and MCG10as proteins contain two domains homologous to an hnRNP KH domain. By generating cell lines that inducibly express either wild-type or mutated forms of MCG10 and MCG10as, we found that MCG10 and MCG10as can induce apoptosis and cell cycle arrest in G_2-M and that both KH domains are necessary for these activities. We also found that MCG10 and MCG10as are capable of binding to poly(C) and that their RNA-binding activity is necessary for inducing apoptosis and cell cycle arrest. These results suggest that the MCG10 RNA-binding protein is a potential mediator of p53 tumor suppression.

MATERIALS AND METHODS

Cell culture and cell lines. HCT116, LS174T, and MCF7 cell lines were purchased from the American Type Culture Collection. RKO, HCT116p53$^{-/-}$, and 80S14 were cultured as described previously (8, 42, 94). RKOE6 and HCT116E6 are derivatives of RKO and HCT116, respectively, that were stably transfected with the E6 gene from human papillomavirus 16 (65). HCT116p53$^{-/-}$ and 80S14 are derivatives of HCT116 in which the genes encoding p53 and p21, respectively, were somatically knocked out (8, 94). The MCF7 cell line, which expresses Tet-VP16 for the generation of tetracycline-inducible cell lines, was purchased from ClonTech (Palo Alto, Calif.). MCF7-p53, an MCF7 derivative that inducibly expresses p53, was generated as previously described (15, 109). p53-3, p53(R249S)-4, and p53(ΔPRD)-5 cell lines, derivatives of H1299 that inducibly express wild-type p53, p53(R249S), and p53(ΔPRD), respectively, were cultured as described previously (15, 108, 109). H1299 cell lines that inducibly express wild-type or mutated forms of MCG10 and MCG10as were generated as previously described (15, 109).

RNA isolation, cDNA subtraction assay, and Northern blot analysis. Polyadenylated RNA was isolated from p53-3 cells using an mRNA purification kit (Pharmacia, Piscataway, N.J.). Total RNA was isolated from cells using Trizol reagents (Life Technologies, Inc., Gaithersburg, Md.). The cDNA subtraction assay was performed using the ClonTech PCR-Select cDNA subtraction kit according to the manufacturer's instruction (ClonTech). Subtracted cDNA fragments were cloned into pCRII vector (Invitrogen, Carlsbad, Calif.). Northern blot analysis was performed as described previously (14, 109). p21 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes were prepared as described previously (109). The *MCG10* probe, a 1.7-kb *Pst*I fragment, was prepared from *MCG10* cDNA.

Plasmids and mutagenesis. The full-length cDNAs for MCG10 and MCG10as were isolated from a cDNA library made with mRNA purified from p53-3 cells and individually cloned into a tetracycline-regulated expression vector, pUHD10-3 (33), between the *Eco*RI and *Xba*I sites. Mutant *MCG10* and *MCG10as* cDNA constructs were generated by PCR and used to replace the corresponding regions of wild-type *MCG10* or *MCG10as* in pUHD10-3. To generate MCG10-ΔKH1, the cDNA fragment encoding amino acids 1 to 188 but lacking amino acids 78 to 185 was amplified using the T3 promoter primer as the 5′-end primer and the 3′-end primer GCA GAT CTG ACT GGC AGG GAT GAC. The resulting fragment was used to replace the corresponding region in *MCG10* between the *Eco*RI and *Bgl*II sites. To generate MCG10-ΔKH2 and MCG10as-ΔKH2, the cDNA fragment encoding amino acids 278 to 424 but lacking amino acids 281 to 329 of MCG10 was amplified by the 5′-end primer ATC GGG CGC CAT GTC ACC ATC ACT and the 3′-end primer TAG GAT CCG GTC GCT GAG AAT AT. The resulting cDNA fragment was used to replace the corresponding region in *MCG10* or *MCG10as* between the *Kas*I and *Bam*HI sites. To generate MCG10as-KH2$^-$ (Ile230Asp), the cDNA fragment encoding amino acids 224 to 369 of MCG10as was amplified by the 5′-end primer CGG GCG CCA GGG CAG CAA GAA CAG CGA G and the 3′-end primer TAG GAT CCG GTC GCT GAG AAT AT. The resulting fragment was used to replace the corresponding region in *MCG10as* between the *Nar*I and *Bam*HI sites.

Antibody production and Western blot analysis. To generate anti-MCG10 antibody, a 1,530-bp *Pst*I-*Nco*I cDNA fragment encoding amino acids 10 to 424 of the MCG10 polypeptide was inserted in frame into the pRSET expression vector (Invitrogen). The His-tagged MCG10 protein was produced in bacteria and purified with Ni-agarose beads. Anti-MCG10 antibody was raised in a rabbit and affinity purified using the His-tagged MCG10 protein (14). For Western blot analysis, cells were collected from culture plates in phosphate-buffered saline (PBS), resuspended in 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and boiled for 5 min. Western blot analysis was performed as previously described (109). Antiactin antibody was purchased from Sigma (St. Louis, Mo.).

Luciferase assay. A 28-bp fragment (5′AGCTTGGTCTTGGCCCAGACTT AGCACA3′) that contains the potential p53-responsive element 1, a 36-bp fragment (5′AGCTTGAACTTAAGACCGAGGCTCTGGACAAGTTGA3′) that contains the potential p53-responsive element 2, and a 27-bp fragment (5′AGCTTGCTCTAGTTCTGGCCATGTTCA3′) that contains the potential p53-responsive element 3 were synthesized and cloned upstream of a minimal c-*fos* promoter and a firefly luciferase reporter gene (41). The resulting constructs were designated p53RE-1, p53RE-2, and p53RE-3, respectively. To mutate the p53-responsive elements in the *MCG10* gene, four nucleotides in p53RE-1 (5′AGCTTGGTaTTtGCCCAGAaTTAtCACA3′) and p53RE-2 (5′A GCTTGAAaTTAAtACCGAGGCTCTGGAaAAtTTGA3′) which are predicted to be critical for p53 binding (shown in lowercase) were replaced. We then generated two reporter vectors, designated m-p53RE-1 and m-p53RE-2, and 2 μg of p53RE-1, m-p53RE-1, p53RE-2, m-p53RE-2, or p53RE-3 was cotransfected into H1299 cells with 1 μg of pcDNA3 control vector or a vector that expresses wild-type or mutant p53. Then 0.1 μg of *Renilla* luciferase assay vector pRL-CMV (Promega, Madison, Wis.) was also cotransfected as an internal control. The dual luciferase assay was performed according to the manufacturer's instructions (Promega).

EMSA. The electrophoretic mobility shift assay (EMSA) probes were 28-bp (p53RE-1) and 36-bp (p53RE-2) oligonucleotides containing a potential p53-responsive element in the *MCG10* gene. The labeled probe DNA (5 ng) was added to a mixture [20 mM HEPES (pH 7.9), 25 mM KCl, 0.1 mM EDTA, 10% glycerol, 2 mM $MgCl_2$, 2 mM spermidine, 0.5 mM dithiothreitol, 0.025% NP-40, 100 ng of double-stranded poly(dI·dC), and 2 μg of bovine serum albumin containing 20 ng of p53 protein. The p53 protein was expressed in a baculovirus expression system and affinity purified using anti-p53 monoclonal antibody Pab421. The p53-DNA complex was resolved in a 4% polyacrylamide gel. For supershifting the p53-DNA complex, 1 μg of anti-p53 monoclonal antibody Pab1801 was added to the reaction. For competition assays, the unlabeled wild-type RGC (20 and 100 ng) or probe DNA (20 and 100 ng) was added to the reaction.

Growth rate analysis and trypan blue exclusion assay. To determine the rate of cell growth, cells were seeded at approximately 5×10^4 to 7.5×10^4 cells per 60-mm plate with or without tetracycline (1.0 μg/ml). The medium was replaced every 72 h. At the times indicated, two plates were rinsed with PBS twice to remove dead cells and debris. Live cells on the plates were trypsinized and collected separately. Cells from each plate were counted at least three times using the Coulter cell counter. The average number of cells from two plates was used for growth rate determination. For the trypan blue dye exclusion assay, all cells were collected separately from two plates at the times indicated. The cells were stained with trypan blue (Sigma) for 10 min. The stained (dead) and unstained (live) cells were counted at least three times using a hemocytometer. The percentage of dead cells was used as an index for the degree of apoptosis.

DNA histogram analysis and annexin V staining assay. Cells were seeded at 2×10^5 per 90-mm plate with or without tetracycline. For DNA histogram analysis, both floating dead cells in the medium and live cells on the plate were collected and fixed with 2 ml of 70% ethanol for at least 30 min. The fixed cells were centrifuged and resuspended in 1 ml of PBS solution containing 50 μg each of RNase A (Sigma) and propidium iodide (Sigma) per ml. The stained cells were analyzed in a fluorescence-activated cell sorter within 4 h. The percentages

of cells in the sub-G_1, G_0-G_1, S, and G_2-M phases were determined using the ModFit program. For the annexin V staining assay, both dead and live cells were collected and washed twice with cold PBS. The cells were resuspended in 0.1 ml of annexin V binding buffer to a density of 10^6/ml and stained according to the manufacturer's instructions (Boehringer, Mannheim, Germany).

Mitochondrial membrane potential assay. To determine whether the cell death mediated by MCG10 goes through the mitochondrial pathway, cells were seeded at approximately 6×10^3 cells/chamber (Fisher Scientific) with or without tetracycline (2 μg/ml) for 3 days. Cells were then rinsed with PBS and stained with ApoAlert Mitochondrial Membrane Sensor reagents according to the manufacturer's instructions (ClonTech). In normal cells, Mitosensor, a cationic dye, is taken up in the mitochondria, where it forms aggregates and exhibits red fluorescence. In apoptotic cells, Mitosensor cannot aggregate in the mitochondria because of altered mitochondrial potentials. As a result, the dye remains in monomeric form in the cytoplasm, where it fluoresces green.

Caspase activity assay. Cells were seeded at approximately 3×10^5 to 5×10^5 per 90-mm plate with or without tetracycline for 3 days. Cells were then rinsed with cold PBS, and caspase activity was assayed using the caspase 3 or 6 colorimetric protease assay reagent according to the manufacturer's instructions (Chemicon International, Inc.). The percent increase in relative caspase activity was the activity in cells expressing p53 or MCG10 divided by that in control cells.

Ribonucleotide homopolymer binding assay. The RNA-binding assay was performed as previously described with modifications (84). Briefly, cells were collected, washed two times with cold PBS, and resuspended in 1 ml of RNA-binding buffer (50 mM Tris-HCl [pH 7.4], 100 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.5% NP-40, 0.5% aprotinin, 2 μg of leupeptin per ml, and 0.5 mM phenylmethylsulfonyl fluoride). Cytoplasmic and nuclear extracts were prepared as previously described (77). For the RNA-binding assay, 0.8 ml of cytoplasmic extracts or nuclear extracts was mixed with 0.2 ml of 5 M NaCl and 5 mg of ribonucleotide homopolymer [poly(A), poly(U), poly(G), or poly(C)]-agarose beads. The mixtures were incubated and rocked at room temperature for 20 min. The beads in the mixture were pelleted and washed three times with RNA-binding buffer. RNA-binding proteins on the beads were resuspended in 2× SDS-PAGE sample buffer, boiled for 8 min, and assayed by Western blot analysis with anti-MCG10 polyclonal antibody.

Nucleotide sequence accession numbers. The human *MCG10* genomic DNA sequence was submitted to GenBank under accession number AF257772. The human *MCG10* and *MCG10as* cDNA sequences were submitted to GenBank under accession numbers AF257770 and AF257771, respectively.

RESULTS

Upregulation of *MCG10* by p53. In our ongoing effort to identify novel p53 target genes, the ClonTech PCR-Select cDNA subtraction assay was performed using mRNA isolated from p53-3, a derivative of the H1299 cell line that inducibly expresses p53 under a tetracycline-regulated promoter (15, 109). Several cDNA fragments that may represent genes induced by p53 were isolated. Among these is *MCG10*, which is a novel gene and encodes a protein with two regions homologous to the KH domain of the hnRNP K protein. To confirm that *MCG10* can be induced by p53, Northern blot analysis was performed using *MCG10* cDNA as the probe. We found that *MCG10* was induced in p53-3 cells when p53 was expressed (Fig. 1A, upper panel, compare lanes 1 and 2). As a control, we tested the expression of three well-defined cellular p53 target genes, p21, GADD45, and 14-3-3σ (28, 35, 42). We found that these genes were also induced by p53 (Fig. 1A, lower panel). The level of induction for *MCG10* was higher than that for GADD45 and 14-3-3σ, albeit lower than that for p21. In addition, mutant p53(R249S) was incapable of activating *MCG10*, p21, GADD45, or 14-3-3σ (Fig. 1A, compare lanes 3 and 4), consistent with the fact that this tumor-derived p53 mutant is defective in transactivation (30). We and others have shown recently that p53(ΔPRD), which lacks the proline-rich domain, is deficient in inducing apoptosis and certain p53 target genes (91, 108). Here we found that p53(ΔPRD) is deficient in inducing *MCG10* (Fig. 1B, compare lanes 3 and 4), suggesting that *MCG10* is a potential mediator of p53-dependent apoptosis (see more below). Furthermore, we determined the kinetics for p53 induction of *MCG10* (Fig. 1C). We found that enhanced expression of *MCG10* was detected as early as 6 h after p53 induction and that maximum induction occurred at 18 and 24 h. Induction of p21 showed similar kinetics.

DNA damage stabilizes and activates p53, leading to induction of p53 target genes (32, 46, 52). If *MCG10* is a true p53 target, it would be induced by DNA damage in cells that contain an endogenous wild-type p53 gene but not in cells that are p53-null. To this end, we tested eight cell lines using the DNA-damaging agent camptothecin, which is an inhibitor of topoisomerase I and can induce double-strand DNA breaks (68). These cells were treated with camptothecin, and the levels of *MCG10* and p21 transcripts were determined by Northern blot analysis (Fig. 1D). We found that both *MCG10* and p21 were induced in camptothecin-treated RKO, HCT116, LS174T, and MCF7 cells, which all contain wild-type p53 (Fig. 1D, lanes 3, 4, 7 to 10, and 13 to 16). Although p21 was not expressed in p21-null 80S14 cells (94), *MCG10* was still induced by DNA damage (Fig. 1D, lanes 9 and 10), indicating that p53 can induce *MCG10* independently of p21. In contrast, *MCG10* was not induced in p53-knockout cells (HCT116p53$^{-/-}$) (Fig. 1D, lanes 5 and 6) or p53-null-like cells (RKOE6 and HCT116E6) (Fig. 1D, lanes 1 and 2 and 11 and 12).

Since exogenous p53 in H1299 cells and endogenous p53 in MCF7 cells are capable of inducing *MCG10*, we wanted to determine whether *MCG10* can be cooperatively induced when both endogenous and exogenous p53s are expressed. To do this, we generated an MCF7 cell line, MCF7-p53, that inducibly expresses HA-tagged p53 under a tetracycline-regulated promoter. We found that *MCG10* was induced in MCF7-p53 cells treated with camptothecin to induce endogenous p53 (Fig. 1E, lane 2) or induced to express exogenous HA-tagged p53 (Fig. 1E, lane 4). In contrast, when both endogenous and exogenous p53s were expressed, the level of *MCG10* induction (6-fold) was more than additive to that induced by endogenous (1.8-fold) or exogenous (3.5-fold) p53 individually (Fig. 1E, compare lane 3 with lanes 2 and 4).

Identification of two potential p53-responsive elements in the *MCG10* gene. To determine whether *MCG10* is a true target of p53, we needed to look for a p53-responsive element in the genomic DNA sequence of the *MCG10* gene. To do this, we screened a human bacterial artificial chromosome library and identified a genomic clone containing *MCG10*. We then sequenced a region of 7,083 nucleotides that spans the entire *MCG10* gene locus (Fig. 2A). We found three potential p53-binding sites, p53-responsive elements 1, 2, and 3, located approximately 900, 1,800, and 2,000 nucleotides upstream of the *MCG10* transcription start site, respectively (Fig. 2A). All three sequences (p53RE-1, GAA CTTAAG aCC GAGGC TCT GGA CAAG TTg; p53RE-2, GGt CTTG gCC C AGA CTTAG CaC; and p53RE-3, Gct CTAG TTC T GGc CATG TTC) contain mismatches (in lowercase) in the noncritical positions within the consensus p53-binding site. Recently, an 81,512-bp genomic DNA sequence from a P1 artificial chromosome clone that contains the *MCG10* gene locus was deposited in GenBank (AC006255). The P1 clone was mapped at chromosome 3p21, a region highly susceptible to aberrant chromosomal rearrangements and deletions in neoplasia (61).

To determine whether these binding sites are responsive to p53 in vivo, three fragments that contain these potential p53-responsive elements (see Materials and Methods) were synthesized and cloned upstream of a minimal c-*fos* promoter and a luciferase reporter gene. The resulting reporter vectors were designated p53RE-1, p53RE-2, and p53RE-3. Each of the reporter vectors was cotransfected into H1299 cells with either pcDNA3 control vector or a vector that expresses wild-type p53 or mutant p53(R175H). The *Renilla* luciferase assay vector

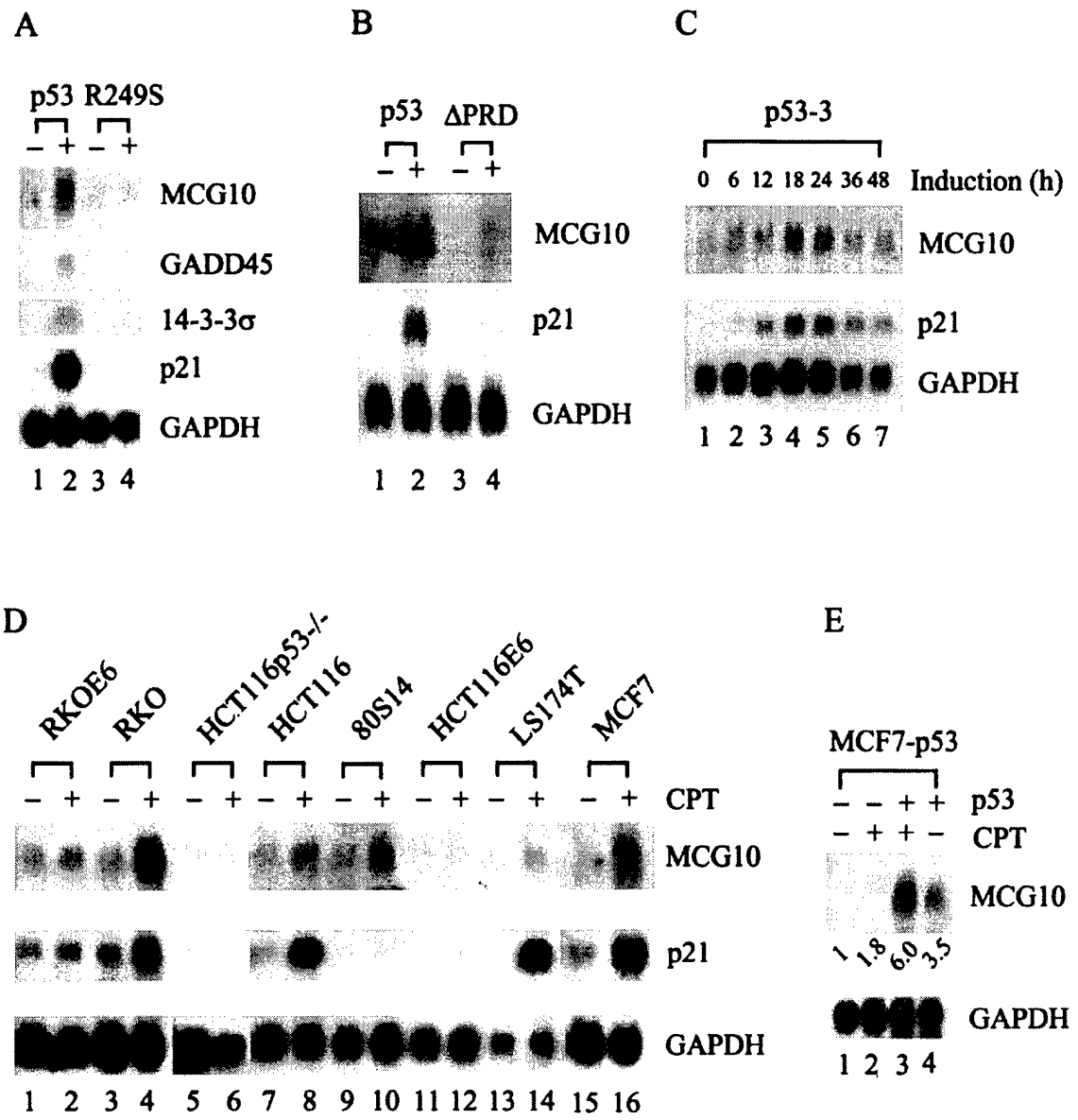
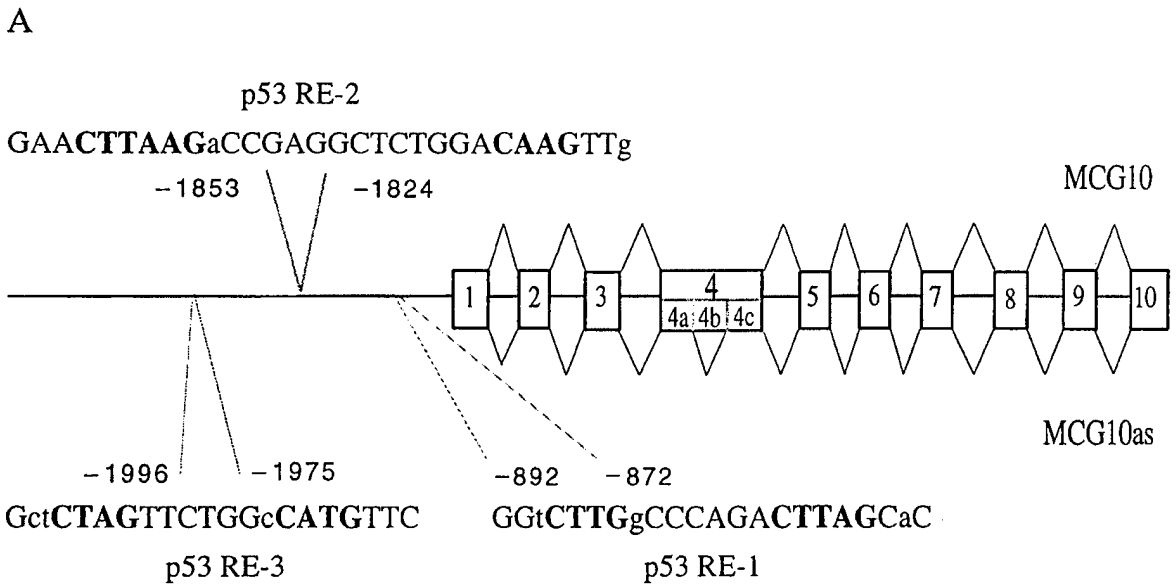


FIG. 1. Upregulation of MCG10 by p53. (A) Wild-type p53 but not mutant p53 induces *MCG10*. Northern blots were prepared using 10 μg of total RNA isolated from p53-3 or p53(R249S) cells that were uninduced (−) or induced (+) to express wild-type p53 or mutant p53(R249S), respectively. The blots were probed with cDNAs derived from the MCG10, 14-3-3σ, GADD45, p21 and GAPDH genes. (B) The apoptosis-deficient deletion mutant p53(ΔPRD) is incapable of inducing *MCG10*. A Northern blot was prepared using 10 μg of total RNA isolated from p53-3 or p53(ΔPRD)-5 cells that were uninduced (−) or induced (+) to express wild-type p53 or mutant p53(ΔPRD), respectively. The blot was probed with *MCG10* cDNA and then reprobed with both p21 and GAPDH cDNAs. (C) Kinetics of p53 induction of *MCG10*. A Northern blot was prepared using 10 μg of total RNA isolated from p53-3 cells that were induced for 0, 6, 12, 18, 24, 36, or 48 h. The blot was probed with *MCG10* cDNA and then reprobed with both p21 and GAPDH cDNAs. (D) *MCG10* is induced by DNA damage in cells that contain an endogenous wild-type p53 gene but not in cells that are functionally p53-null. Northern blots were prepared using 10 μg of total RNA isolated from seven individual cell lines (see text for details) as indicated above the figure, which were untreated (−) or treated (+) with 300 nM camptothecin for 24 h. The blots were probed with cDNAs derived from *MCG10*, p21, and GAPDH. (E) Exogenous inducible p53 cooperates with endogenous wild-type p53 in MCF7 cells to induce *MCG10*. A Northern blot was prepared using 10 μg of total RNA isolated from MCF7-p53 cells that were untreated (lane 1), treated with 300 nM camptothecin (CPT) to induce endogenous wild-type p53 (lane 2), induced to express exogenous p53 and treated with 300 nM camptothecin to induce endogenous wild-type p53 (lane 3), or induced to express exogenous p53 (lane 4). The blot was probed with cDNAs derived from *MCG10* and GAPDH.

pRL-CMV was also cotransfected as an internal control. We found that the luciferase activity of p53RE-1 and p53RE-2 but not p53RE-3 was markedly increased by wild-type p53 (Fig. 2B). Mutant p53(R175H) was incapable of increasing the luciferase activity of p53RE-1 and p53RE-2 (Fig. 2B). We also replaced four nucleotides in p53RE-1 and p53RE-2 predicted to be critical for p53 binding (see Materials and Methods) and generated two reporters, designated m-p53RE-1 and m-p53RE-2. We found that the luciferase activity for both m-p53RE-1 and m-p53RE-2 was not significantly increased by wild-type p53 or mutant p53(R175H) (Fig. 2B). These results suggest that two of the three potential p53-responsive elements in *MCG10* do function in vivo.

To further determine whether p53 binds to the responsive



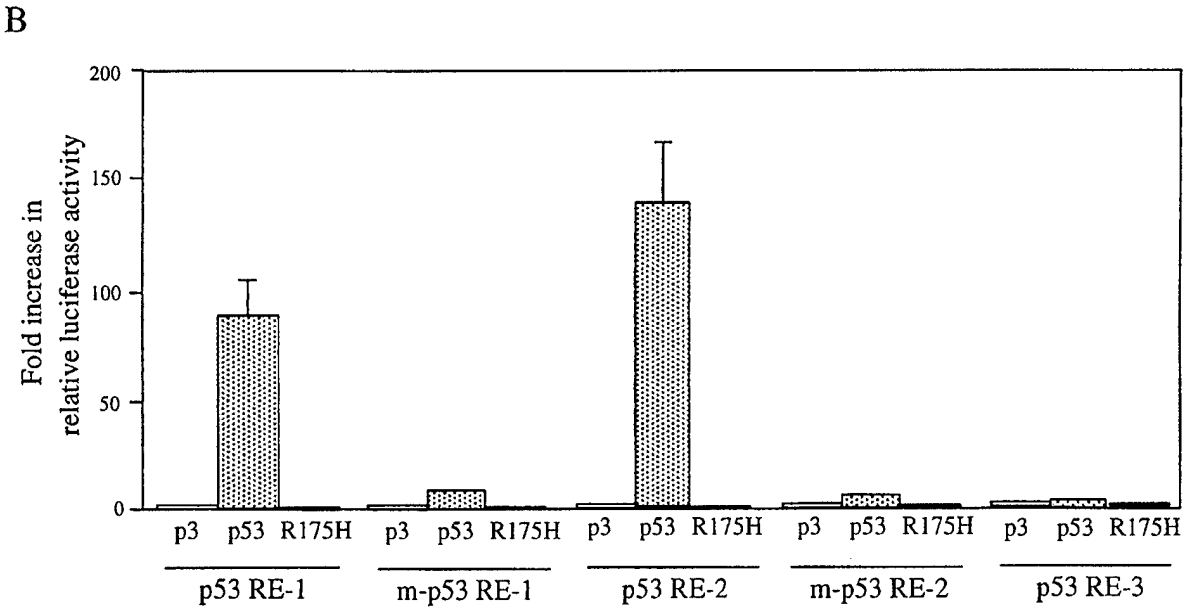


FIG. 2. Identification of two p53-responsive elements in the *MCG10* gene. (A) Schematic representation of the *MCG10* genomic DNA structure. Exons are shown as numbered boxes, and introns are shown as lines. The locations of two potential p53-responsive elements in the promoter of the *MCG10* gene are indicated. Bold uppercase letters represent nucleotides predicted to be critical for the consensus p53-responsive element. Lowercase letters represent mismatches. The transcript for *MCG10* is drawn above the gene structure, and the transcript for *MCG10as* is shown below. Exon 4b is not present in the *MCG10as* transcript. (B) Two of the three potential p53-binding sites but not their mutated forms in the *MCG10* gene are responsive to wild-type p53 in vivo. p53RE-1, m-p53RE-1, p53RE-2, m-p53RE-2, or p53RE-3 (2 μg) was cotransfected into H1299 cells with 1 μg of pcDNA3 control vector or a vector that expresses wild-type p53 or mutant p53(R175H). The fold increase in relative luciferase activity is the luciferase activity induced by p53 divided by that induced by pcDNA3. Error bars represent the standard deviations from at least three experiments. (C) p53 binds specifically to both p53RE-1 and p53RE-2 in vitro. The 28- and 36-bp oligonucleotide fragments containing p53RE-1 and p53RE-2, respectively, in the *MCG10* gene were labeled with [α-^{32}P]dCTP. The labeled probe DNA (5 ng) was added to a mixture containing 20 ng of p53 protein. The p53-DNA complex was resolved in a 4% polyacrylamide gel. For competition assays, 5- or 20-fold excess unlabeled 28-bp probe DNA (lanes 3 and 4), 36-bp unlabeled probe DNA (lanes 9 and 10), or RGC (lanes 5 and 6 and 11 and 12) was added to the reactions.

elements in the *MCG10* gene, two DNA fragments (28 and 36 bp) that contain p53RE-1 and -2 (see Materials and Methods) were synthesized, ^{32}P-labeled, and used in an EMSA (Fig. 2C). We found that when the purified p53 protein was mixed with these DNA fragments, a complex that presumably contained both p53 and p53RE-1 or -2 was detected (Fig. 2C, lanes 2 and 8). The complex was supershifted with the anti-p53 monoclonal antibody Pab1801 (data not shown). We also used the

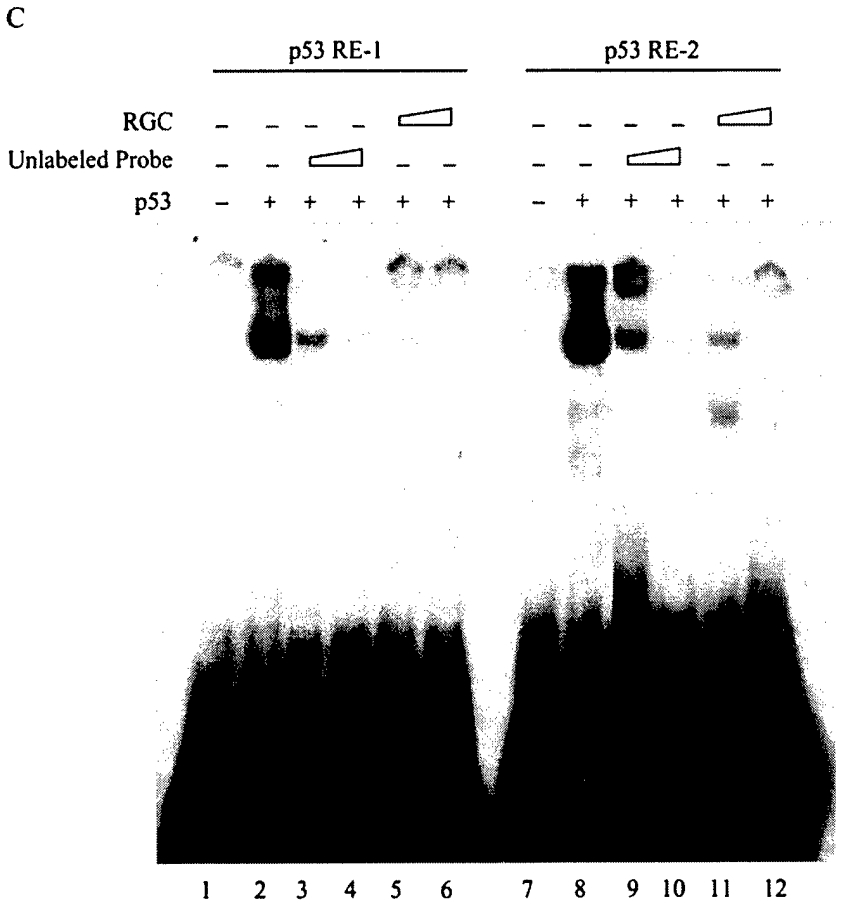


FIG. 2—*Continued.*

unlabeled probe DNA and a fragment that contains a wild-type p53-binding site from the ribosomal gene cluster (RGC) (43) as competitors. The unlabeled probe DNA and wild-type RGC competed with the ^{32}P-labeled 28- and 36-bp probe DNA fragments from the *MCG10* gene and inhibited the formation of the p53-DNA complex in a dose-dependent manner (Fig. 2C, lanes 3 to 6 and 9 to 12). These results indicate that p53 interacts specifically with both p53RE-1 and -2 in the *MCG10* gene.

***MCG10* gene locus encodes at least two alternatively spliced transcripts for novel KH motif RNA-binding proteins.** To analyze the activity of the *MCG10* gene product, we used the 163-bp cDNA fragment from the cDNA subtraction assay to screen a cDNA library made from mRNA isolated from p53-3 cells. Two cDNA clones (2,623 and 2,458 nucleotides) were identified. When both cDNA sequences were aligned with the 7,083-nucleotide genomic DNA sequence, we found that 10 exons encode the 2,623-nucleotide *MCG10* transcript. The 2,458-nucleotide cDNA clone represents an alternatively spliced transcript, *MCG10as*, which lacks 165 nucleotides within exon 4. We refer to the region not expressed in *MCG10as* as exon 4b (see Fig. 2A). The *MCG10* and *MCG10as* transcripts encode novel polypeptides of 424 and 369 amino acids, respectively. Each protein contains two KH domains, three proline-rich domains, one potential nuclear export signal, and one potential nuclear localization signal (Fig. 3A to C). A sequence alignment of the KH domains from hnRNP K, FMR1, MCG10, and MCG10as showed that the critical residues in the KH domains of hnRNP K and FMR1 are conserved in those of MCG10 and MCG10as (Fig. 3D). For example, the GXXG motif within the KH domain of hnRNP K (82) and the critical Ile (at residue 304, marked with an asterisk) in the FMR1 KH2 (66) are conserved in the KH domains of MCG10 and MCG10as.

MCG10 and MCG10as can induce apoptosis and cell cycle arrest in G_2-M. Activation of p53 leads to at least two well-characterized cellular responses, cell cycle arrest and apoptosis (1, 13, 52). Since *MCG10* can be induced by p53, we wanted to determine whether *MCG10* is capable of mediating p53 tumor suppression. To this end, we generated several cell lines that inducibly express *MCG10* and *MCG10as* under the control of a tetracycline-regulated promoter. The levels of the MCG10 and MCG10as proteins in four representative H1299 cell lines were determined by Western blot analysis with anti-MCG10 antibody (Fig. 4A). A 45-kDa polypeptide was specifically recognized by anti-MCG10 antibody in both MCG10- and MCG10as-producing cells when induced. Interestingly, we found that the apparent molecular masses of MCG10 and MCG10as are nearly identical, although the MCG10 polypeptide is 55 amino acids longer than MCG10as (Fig. 3A and B). When the levels of actin protein were normalized in various cells, we found that MCG10 and MCG10as were expressed at comparable levels. We then measured the growth rates of MCG10-17 and MCG10as-10 cells in the absence and presence of MCG10 and MCG10as over a 5-day period. We found that

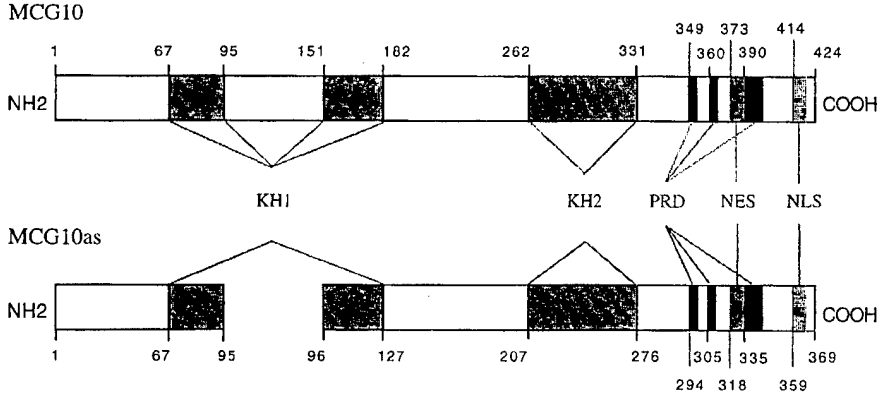
A MCG10

MPRCPALILYLQSSARITISEGSCPERITTITGSTAAVFHAVSMIAFKLDEDL
CAAPANGGNVSRP PVTLRLVIPASQCGSLIGKAGTKIKEIRE*VRGEIYHPQ* KH-1
*GIRGKGAVVRGVLGLWRPPHLESSEPGQPFSGLWEQPEVAPVLCLQ*TTGA
QVQVAGDLLPNSTERAVTVSGVPDAIIL CVRQICAVILESPPKGATIPYHPS
LSLGTVLLSANQGFSVQGQYGAVTPAEVTKLQQLSSHAVPFATPSVVPGL
DPGTQ TSSQEFLVPNDLIGCVIGRQGSKISEIRQMSGAHIKIGNQAEGAGER KH-2
HVTITGSPVSIALAQYLITACLE TAKSTSGGTPSSAPADLPAPFSPPLTALPT
APPGLLGTPYAISLSNFIGLKPMPFLALPPASPGPPPGLAAYTAKMAAANG
SKKAERQKFSPY

B MCG10as

MPRCPALILYLQSSARITISEGSCPERITTITGSTAAVFHAVSMIAFKLDEDLC
AAPANGGNVSRP PVTLRLVIPASQCGSLIGKAGTKIKEIRETTGAQVQVAGD KH-1
LLPNSTERAVTVSGVPDAIIL CVRQICAVILESPPKGATIPYHPSLSLGTVLLS
ANQGFSVQGQYGAVTPAEVTKLQQLSSHAVPFATPSVVPGLDPGTQ TSSQE
FLVPNDLIGCVIGRQGSKISEIRQMSGAHIKIGNQAEGAGERHVTITGSPVSIA KH-2
LAQYLITACLE TAKSTSGGTPSSAPADLPAPFSPPLTALPTAPPGLLGTPYAIS
LSNFIGLKPMPFLALPPASPGPPPGLAAYTAKMAAANGSKKAERQKFSPY

C MCG10



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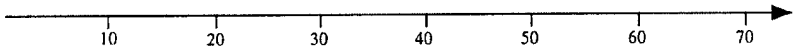


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hnRNP K-KH1     MVELRILLQSKNAGAVIGKGGKNIKALRTD----------------------YN---------------------  32
hnRNP K-KH2     DCELRLLIHQSLAGGIIGVKGAKIKELRE-------------------------------NTQTTIK--------  36
hnRNP K-KH3     IITTQVTIPKDLAGSIIGKGGQRIKQIRHESG-----------------------------------------------  32
FMR1-KH1        RFHEQFIVREDLMGLAIGTHGANIQQARKVPGV-----------------------------------------------  33
FMR1-KH2        FAEDVIQVPRNLVGKVIGKNGKLIQEIVDKSGVVRVRIEAENEKNVPQEEEIMPPNSLPSNNSRV-GPNAPEEK  73
MCG10-KH1       PVTLRLVIPASQCGSLIGKAGTKIKEIREVRG-------------------EIYHPQGIRGKGAVVRGVLGLWRP  56
MCG10-KH2       TSSQEFLVPNDLIGCVIGRQGSKISEIRQMSG-----------------------------------------------  32
MCG10as-KH1     PVTLRLVIPASQCGSLIGKAGTKIKEIRE-----------------------------------------------  29
                              GXXG  *
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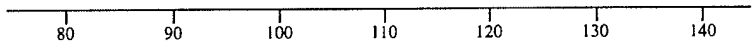


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hnRNP K-KH1     ASVSVPDSSGP-------------------------ERILSISADI---------ETIGEILKKIIPTLE  68
hnRNP K-KH2     --------------LFQEC------CP------HSTDRVVLIGGK---------PDRVVECIKIILDLIS  71
hnRNP K-KH3     ASIKIDEPLEG------------------------SEDRIITITG---------TQDQIQNAQYLLQNSVK  70
FMR1-KH1        TAIDLDEDTCTFH-----------------------------------IYGEDQDAVKKARSF----LE  63
FMR1-KH2        KHLDIKENSTHFSQP-NSTKVQRVLVASSVVAGESQKPELKAWQGMVPFVFVGTKDSIANATVLLDYHLN  142
MCG10-KH1       PHLESSEPGQPFSGLWEQPEVAPVLCL------QTTGAQVQVAGDLLP----NSTERAVTVSGVPDAIIL  116
MCG10-KH2       AHIKIGNQAEG------------------------AGERHVTITG---------SPVSIALAQYLITACLE  70
MCG10as-KH1     ----------------------------------TTGAQVQVAGDLLP----NSTERAVTVSGVPDAIIL  61
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FIG. 3. (A and B) Deduced amino acid sequences of MCG10 and MCG10as. The N-terminal KH domain (KH1) and C-terminal KH domain (KH2) in MCG10 and MCG10as are boxed. The bold italic letters represent a 55-amino-acid insertion in the N-terminal KH domain of MCG10. Three proline-rich domains (PRD) are underlined. The nuclear export signal (NES) and nuclear localization signal (NLS) are marked by dashes. (C) Schematic representations of MCG10 and MCG10as protein structures. The locations of specific features are indicated by the amino acid number. (D) Sequence alignment of eight KH domains from hnRNP K, FMR1, MCG10, and MCG10as. Numbers on the right indicate positions of the ending amino acids in the KH domain. Highly conserved positions are highlighted in colors. The GXXG motif is shown below the alignment. The critical isoleucine residue for FMR1 KH2 that is mutated in fragile X syndrome is indicated (*).

both MCG10 and MCG10as can suppress cell proliferation (Fig. 4B and C).

To determine whether the growth suppression by MCG10 and MCG10as is due to cell cycle arrest, apoptosis, or both, we performed DNA histogram analysis. When cells were induced to express MCG10 for 2, 4, and 6 days, we found that the percentage of cells in S phase decreased from 35 to 23% (Fig. 5A and B), 37 to 29% (Fig. 5E and F), and 35 to 26% (Fig. 5I and J), respectively. In contrast, we found that the percentage of cells in G_2-M phase increased from 14 to 23% (Fig. 5A and B), 15 to 31% (Fig. 5E and F), and 16 to 35% (Fig. 5I and J), respectively. We also found that the number of cells in G_2/M was increased when *MCG10* was induced for 1 day (data not shown). The maximum effect was observed between 2 and 4

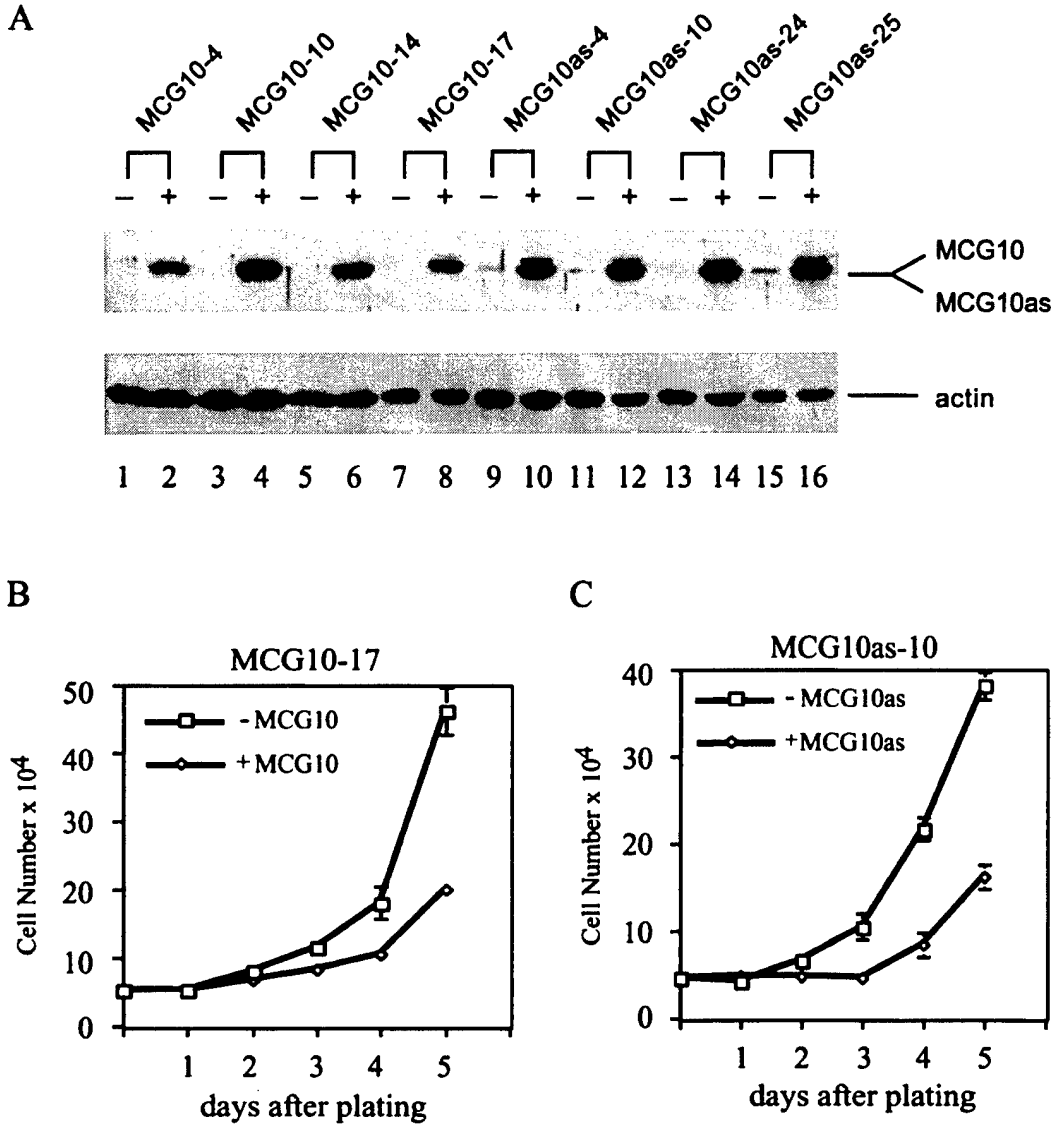

FIG. 4. MCG10 and MCG10as are capable of suppressing cell proliferation. (A) Levels of MCG10, MCG10as, and actin were assayed by Western blot analysis in cell lines that inducibly express MCG10 or MCG10as. Cell extracts were prepared from uninduced cells (−) or cells induced (+) to express MCG10 or MCG10as. The blot was probed with affinity-purified anti-MCG10 polyclonal antibody (upper panel) and then reprobed with antiactin polyclonal antibody (lower panel). (B and C) Growth rates of MCG10-17 and MCG10as-10 cells in the presence (◇) or absence (□) of MCG10 or MCG10as, respectively, were measured as described in Materials and Methods. Error bars represent the standard deviations from at least three experiments.

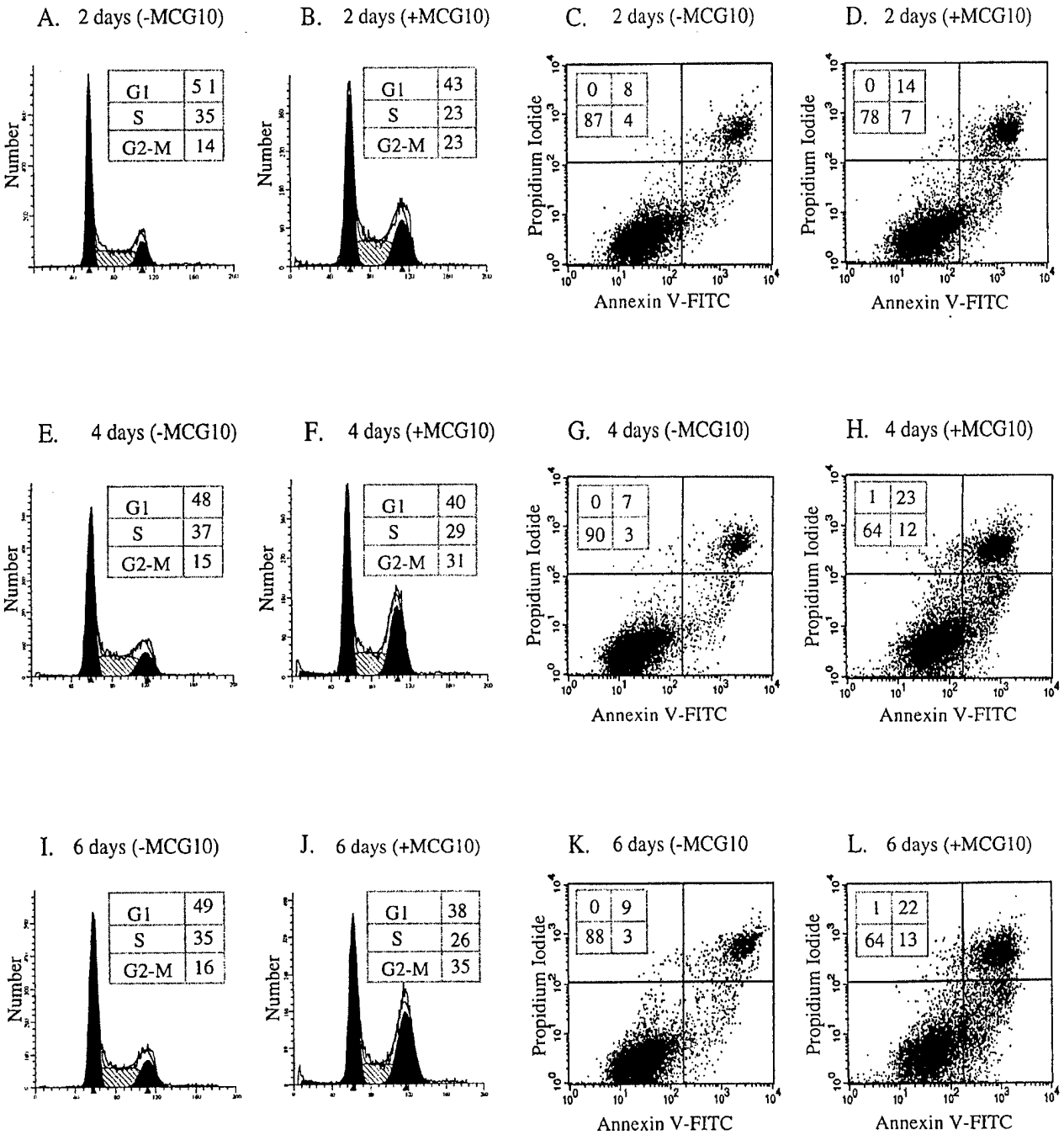

FIG. 5. MCG10 is capable of inducing cell cycle arrest in G_2-M and apoptosis. DNA content was quantitated by propidium iodide staining of fixed cells that were uninduced (−MCG10) or induced (+MCG10) to express MCG10 for 2 days (A and B), 4 days (E and F), and 6 days (I and J). Apoptotic cells were quantitated by propidium iodide-annexin V staining of cells that were uninduced (−MCG10) or induced (+MCG10) to express MCG10 for 2 days (C and D), 4 days (G and H), and 6 days (K and L).

days following induction of *MCG10*. This is consistent with the fact that p53-mediated cell cycle arrest occurs within 24 h but remains incomplete till 48 h (15). Furthermore, we found that the ability of MCG10 to induce arrest in G_2/M is higher than that of p53 in H1299 cells, although slightly lower than that of GADD45 (95, 108). These results suggest that MCG10 can induce cell cycle arrest in G_2-M. However, no substantial increase was detected for cells in sub-G_1. Since cells can undergo apoptosis without DNA fragmentation (67, 71, 80), we determined whether MCG10 can induce cell death by the annexin V staining assay. We found that when cells were induced to express MCG10 for 2, 4, and 6 days, the percentage of stained cells (a combination of cells in both the upper right and lower right boxes) was increased from 12 to 21% (Fig. 5C and D), 10 to 35% (Fig. 5G and H), and 12 to 35% (Fig. 5K and L), respectively.

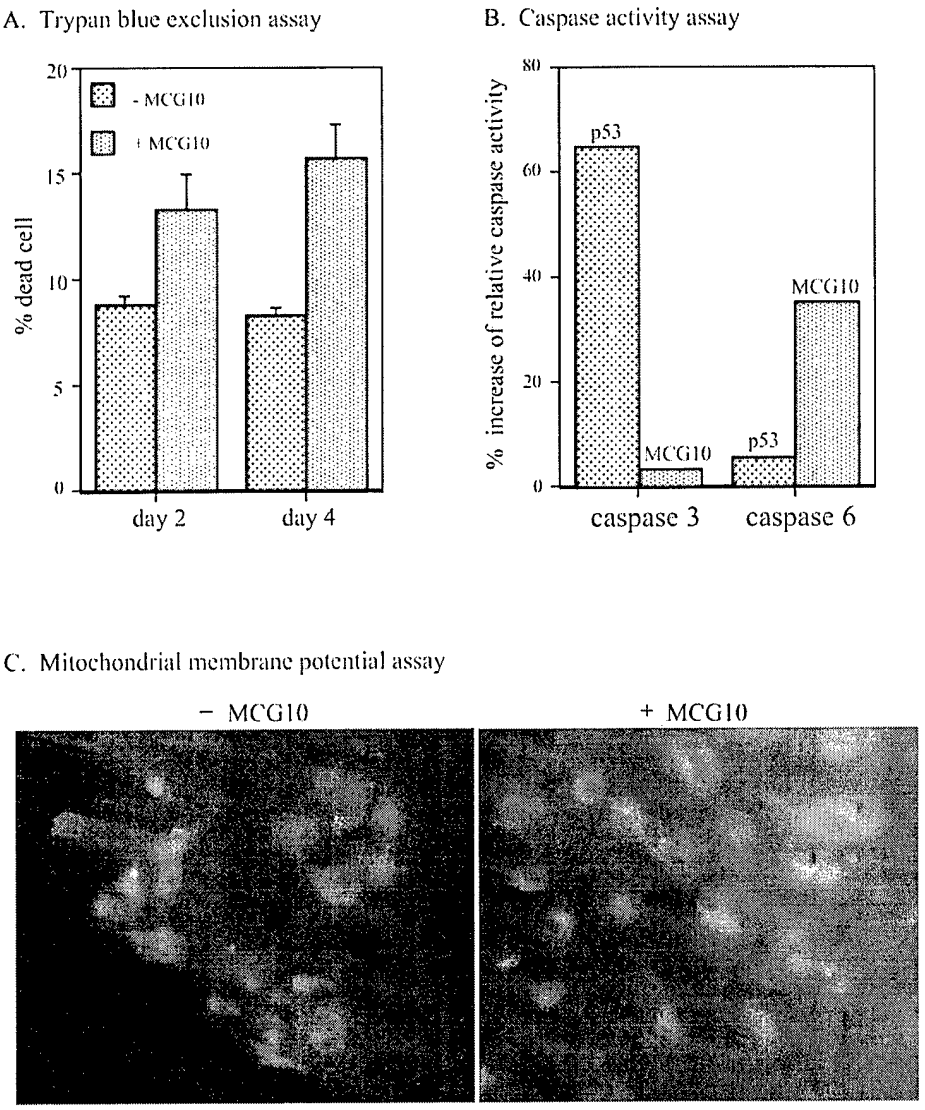

FIG. 6. MCG10 activates caspase 6 and induces apoptosis through the mitochondrial pathway. (A) The percentage of dead cells induced by MCG10 was quantified by trypan blue dye exclusion. Cells were seeded in the presence (+) or absence (−) of MCG10 for 2 or 4 days. Both unstained and trypan blue-stained cells were counted using a hemocytometer. Error bars represent the standard deviations from at least three experiments. (B) Caspase 6 is activated by MCG10. p53-3 or MCG10-17 cells were uninduced or induced to express p53 or MCG10 for 3 days. Cells were then collected and assayed for the activity of caspases 3 and 6 as described in Materials and Methods. (C) The mitochondrial membrane potentials were altered in cells induced to express MCG10. MCG10-17 cells were uninduced (−MCG10) or induced to express MCG10 (+MCG10) for 3 days, stained with Mitosensor, and analyzed by fluorescence microscopy.

To further demonstrate that MCG10 can induce apoptosis, we performed a trypan blue dye exclusion assay. We found that the percentage of dead (trypan blue stained) cells was significantly increased in cells induced to express MCG10 for 2 and 4 days (Fig. 6A). It is well established that during the apoptotic cascade, several caspases are activated and the mitochondrial membrane potential of apoptotic cells is altered (67). Therefore, we analyzed the activity of caspases 3 and 6 and the mitochondrial membrane potential in cells with and without induction of MCG10. We found that the activity of caspase 6 but not caspase 3 was significantly increased by MCG10 (Fig. 6B). We also found that p53 substantially activated caspase 3 and, to a lesser extent, caspase 6 (Fig. 6B). Furthermore, the mitochondrial membrane was not permeable to Mitosensor, a cationic dye in cells expressing MCG10 (Fig. 6C), or p53 (data not shown), suggesting that the mitochondrial membrane potential is altered. Similar results were obtained for MCG10as-producing cells (Fig. 7). These results suggest that MCG10 can induce apoptosis without causing DNA fragmentation.

Role of the KH domain in the activity of MCG10 and MCG10as. To determine whether the KH domain is necessary for the ability of MCG10 and MCG10as to induce cell cycle arrest and apoptosis, we constructed three KH domain deletion mutants, MCG10-ΔKH1, MCG10-ΔKH2, and MCG10as-ΔKH2. We then generated several cell lines that inducibly express these mutants. Expression of the mutant MCG10 and MCG10as proteins was assayed in Western blots using anti-MCG10 antibody (Fig. 8A, C, and E). Levels of the mutant proteins in MCG10-ΔKH1-5 and MCG10-ΔKH2-20 were fairly comparable to that in MCG10-17 cells (Fig. 8A and C). The level of the mutant protein expressed in MCG10as-ΔKH2-23 cells was relatively low compared to that in MCG10as-10 cells

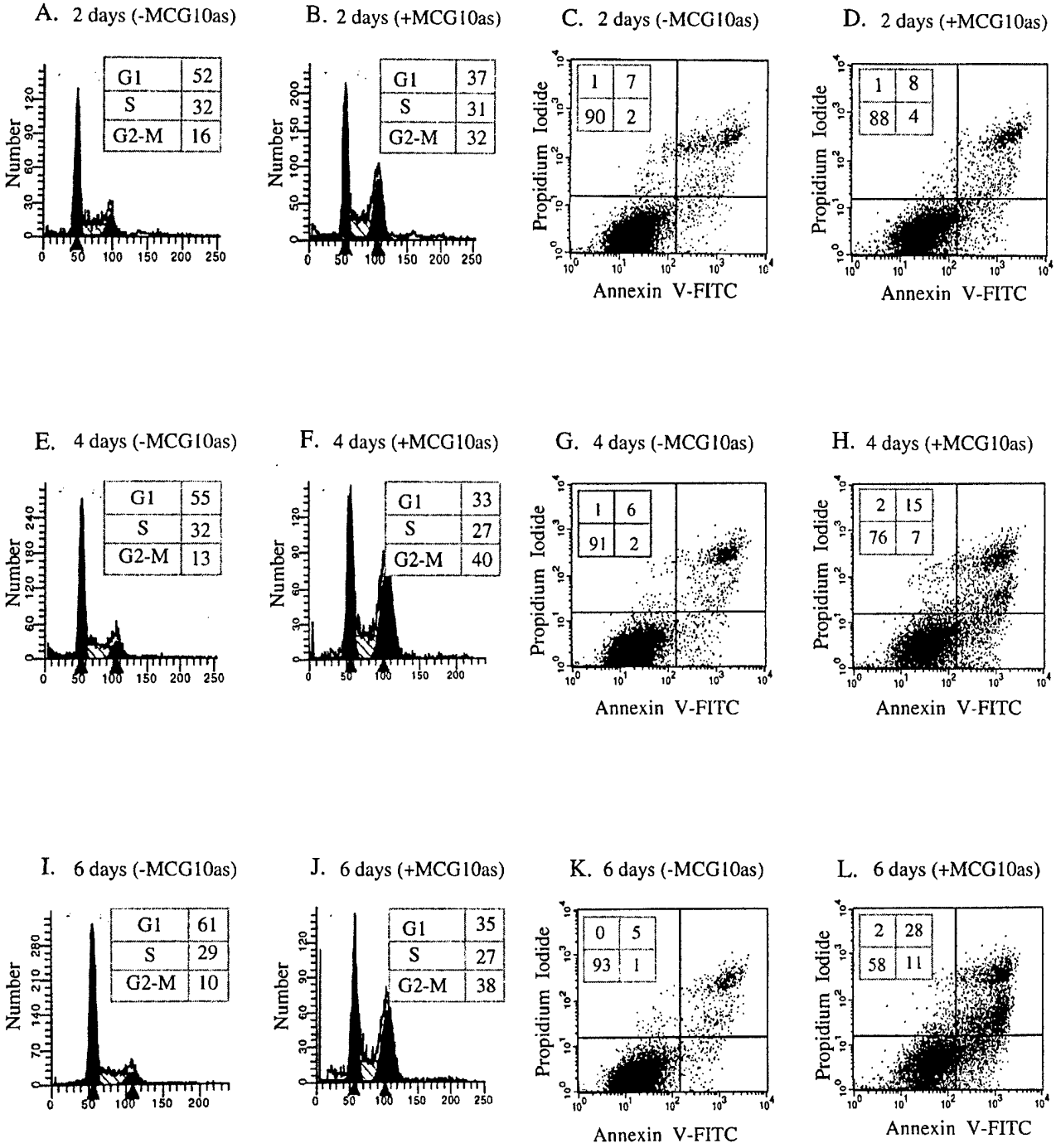

FIG. 7. MCG10as is capable of inducing both cell cycle arrest in G_2-M and apoptosis. DNA content was quantitated by propidium iodide staining of fixed cells that were uninduced (−MCG10as) or induced (+MCG10as) to express MCG10as for 2 days (A and B), 4 days (E and F), and 6 days (I and J). Apoptotic cells were quantitated by propidium iodide-annexin V staining of cells that were uninduced (−MCG10as) or induced (+MCG10as) to express MCG10as for 2 days (C and D), 4 days (G and H), and 6 days (K and L).

(data not shown). We then measured the growth rates of MCG10-ΔKH1-5, MCG10-ΔKH2-20, and MCG10as-ΔKH2-23 cells in the absence and presence of protein induction over a 5-day period. We found that none of the mutants were capable of suppressing cell proliferation (Fig. 8B, D, and F). Since a single KH domain remains in each mutant, the results suggest that both KH domains are required for the activity of MCG10 and MCG10as.

Both KH domains in MCG10 and MCG10as are necessary for binding RNA. MCG10 and MCG10as each contain two KH domains. Since KH domains are known to bind RNA, we wanted to determine whether the KH domains in MCG10 and

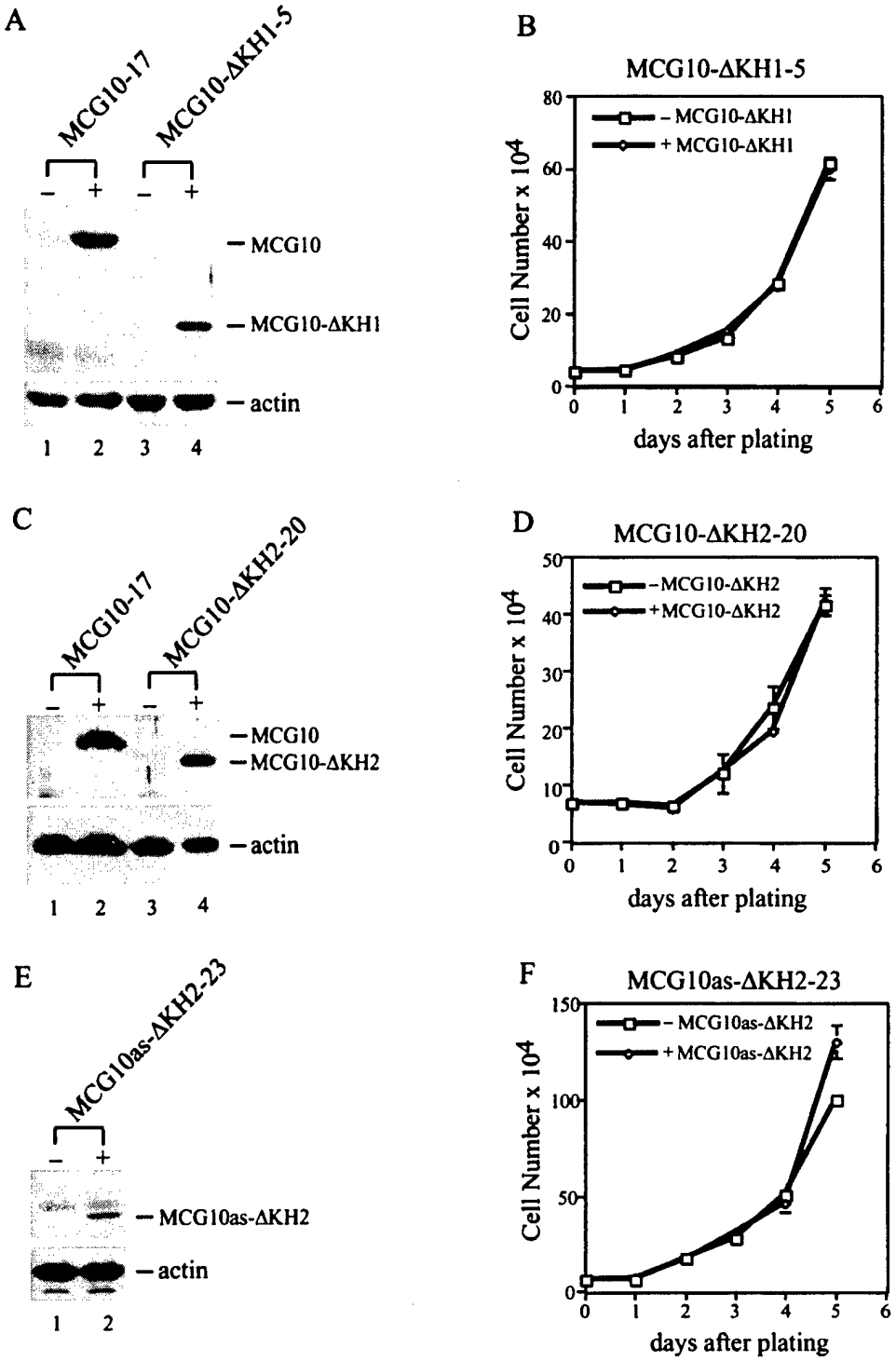

FIG. 8. Both KH domains in MCG10 and MCG10as are necessary for inducing cell cycle arrest and apoptosis. (A) Levels of MCG10 and actin in MCG10-17 and MCG10-ΔKH1-5 cell lines were assayed by Western blot analysis. Cell extracts were prepared from uninduced cells (−) or cells induced (+) to express MCG10 or MCG10-ΔKH1. The blot was probed with affinity-purified anti-MCG10 polyclonal antibody (upper panel) and then reprobed with antiactin polyclonal antibody (lower panel). (B) Growth rates of MCG10-ΔKH1-5 cells in the presence (◇) and absence (□) of MCG10-ΔKH1 were measured as described in Materials and Methods. (C) Levels of MCG10 and actin in MCG10-17 and MCG10-ΔKH2-20 cell lines were assayed by Western blot analysis as described for panel A. (D) Growth rates of MCG10-ΔKH2-20 cells in the presence (◇) and absence (□) of MCG10-ΔKH2. (E) Levels of MCG10as-ΔKH2 and actin in the MCG10as-ΔKH2-23 cell line were assayed by Western blot analysis as described for panel A. (F) Growth rates of MCG10as-ΔKH2-23 cells in the presence (◇) and absence (□) of MCG10as-ΔKH2. Error bars represent the standard deviations from at least three experiments.

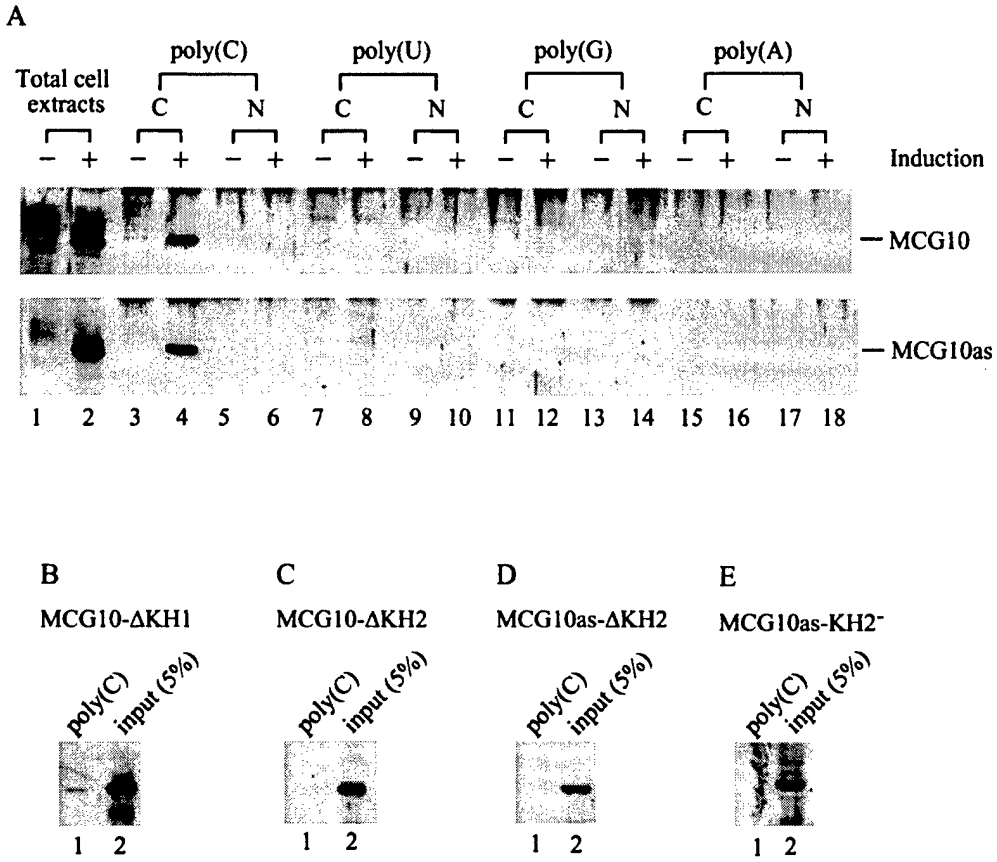

FIG. 9. KH domain in MCG10 and MCG10as is capable of and necessary for binding poly(C). (A) MCG10 and MCG10as can bind to poly(C) but not to poly(U), poly(G), or poly(A). Total cell extracts run in lanes 1 and 2 were prepared from MCG10-17 and MCG10as-10 cells that were uninduced (−) and induced (+) to express MCG10 (upper panel) or MCG10as (lower panel). Cytoplasmic extracts (C) and nuclear extracts (N) were prepared from uninduced cells (−) or cells induced (+) to express MCG10 or MCG10as and mixed with poly(C)-, poly(U)-, poly(G)-, or poly(A)-agarose beads. Proteins bound to the beads were isolated and assayed by Western blot analysis using anti-MCG10 antibody. (B) The KH1 domain in MCG10 is necessary for binding poly(C). Cytoplasmic extracts were prepared from cells induced to express MCG10-ΔKH1, and the RNA-binding assay was performed as described for panel A. (C and D) The KH2 domain in MCG10 and MCG10as is necessary for binding poly(C). Cytoplasmic extracts were prepared from cells induced to express MCG10-ΔKH2 or MCG10as-ΔKH2, and the RNA-binding assay was performed. (E) A point mutation (Ile230Asp) in the KH2 domain abrogates the ability of MCG10as to bind to poly(C). Cytoplasmic extracts were prepared from cells induced to express MCG10as-KH2⁻, and the RNA-binding assay was performed.

MCG10as also bind to RNA. To do this, poly(C)-, poly(G)-, poly(U)-, or poly(A)-agarose beads were added to cytoplasmic or nuclear extracts purified from uninduced cells or cells induced to express MCG10 or MCG10as. Proteins that specifically bound to the homopolymer beads were isolated, and the MCG10 and MCG10as proteins were identified by Western blot analysis. We found that MCG10 and MCG10as can bind to poly(C) but not to poly(A), poly(U), or poly(G) (Fig. 9A). This is consistent with the RNA-binding specificity for the KH domain (48, 74, 82). We did not detect any MCG10 and MCG10as in the nuclear extracts, suggesting that these proteins are predominantly located in the cytoplasm.

To determine whether the KH domain deletion mutants that are defective in suppressing cell proliferation are also inert in binding RNA, the poly(C) RNA-binding assay was performed using cytoplasmic extracts from cells expressing MCG10-ΔKH1, MCG10-ΔKH2, and MCG10as-ΔKH2. We found that MCG10-ΔKH2 and MCG10as-ΔKH2 were incapable of binding poly(C) (Fig. 9C and D), whereas MCG10-ΔKH1 bound poly(C) extremely weakly (Fig. 9B). It has been reported that a missense mutation from Ile to Asp at residue 304 in KH2 of FMR1 abrogates its RNA-binding activity (66). To determine whether such a mutation would affect the RNA-binding activity of MCG10as, we generated a cell line that inducibly expresses the analogous mutant, designated MCG10as-KH2⁻. We found that, like the FMR1 mutant, MCG10as-KH2⁻ was defective in binding RNA (Fig. 9E).

Poly(C)-binding MCG10 protein level is increased in cells following DNA damage in a p53-dependent manner. We have shown above that the *MCG10* gene is induced by p53 and DNA damage (Fig. 1). To determine whether the level of MCG10 protein is increased in cells following a genotoxic stress, cytoplasmic cell extracts were prepared from RKO, RKOE6, HCT116, and HCT116E6 cells that were untreated or treated with 300 nM camptothecin for 24 h. MCG10 was isolated using the poly(C) beads and assayed by Western blot analysis with anti-MCG10 antibody. We found that the level of MCG10 protein was increased nearly 11-fold in RKO cells (Fig. 10, compare lanes 1 and 2), but only 2.6-fold in RKOE6 cells that are functionally p53-null when treated with camptothecin (Fig. 10, compare lanes 3 and 4). In addition, MCG10 was detected in HCT116 cells only when treated with camptothecin, but not in HCT116E6 cells, which are functionally p53-null (Fig. 10, lanes 5 to 8). A nonspecific protein that migrated slightly slower than MCG10 was detected in HCT116 cells (lanes 5 and 6). These results are consistent with the data obtained by

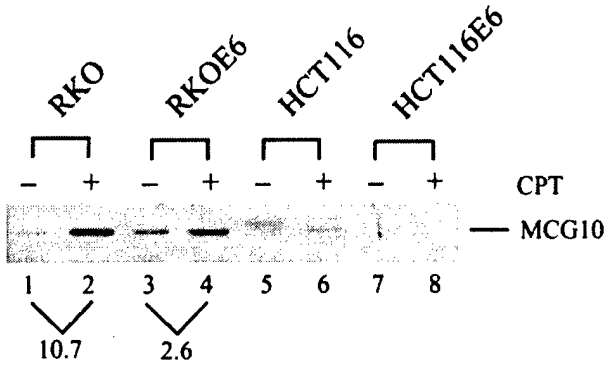

FIG. 10. Level of the poly(C)-binding MCG10 protein is increased in cells treated with DNA-damaging agent camptothecin in a p53-dependent manner. Cytoplasmic extracts were prepared from RKO, RKOE6, HCT116, and HCT116E6 cells that were untreated (−) or treated (+) with camptothecin (CPT). The RNA-binding assay was performed as described in the legend to Fig. 9A.

Northern blot analysis (Fig. 1C) that induction of MCG10 by DNA damage is p53 dependent. It should be noted that, although *MCG10* mRNA is not induced by DNA damage in RKOE6 cells (Fig. 1C, lanes 1 and 2), the level of poly(C)-binding MCG10 protein is increased, albeit to a lesser extent than in RKO cells. This suggests that MCG10 can be regulated posttranscriptionally by DNA damage in a p53-independent manner.

DISCUSSION

RNA-binding proteins have diverse functions in the regulation of gene expression. This is the first report, to our knowledge, that a KH motif RNA-binding protein is regulated by p53 and that it serves as a mediator in inducing apoptosis and cell cycle arrest in G_2-M. We have demonstrated that deletion of either of the KH domains or a point mutation in the C-terminal KH domain of MCG10as abrogates or severely diminishes the activity of MCG10 and MCG10as in binding RNA. As a result, the MCG10 and MCG10as mutants defective in RNA binding are also defective in inducing apoptosis and cell cycle arrest. These results indicate that, like other RNA-binding proteins, the RNA-binding activity is critical for the function of MCG10 and MCG10as. Interestingly, a 55-amino-acid insertion in the N-terminal KH domain does not interfere with the RNA-binding activity of MCG10.

Previously, we and others have shown that p53 cellular target genes are differentially regulated by p73 (21, 50, 107). We found that the *MCG10* gene is among the group that is not induced by p73, further supporting the idea that the p73 signaling pathway is different from that for p53 (13). It should be mentioned that, like other p53 target genes, the *MCG10* gene is induced by DNA damage in a p53-dependent manner (Fig. 1). DNA-damaging agents can induce a number of DNA-binding proteins by both transcriptional and posttranscriptional mechanisms, such as p53 (47), c-*jun* (106), and c-*fos* (29). However, the role of RNA-binding proteins in response to genotoxic stresses is mostly unexplored. A18 hnRNP, which contains one each of the RBD and RGG RNA-binding motifs, can be induced in response to UV-induced DNA damage (81). Nevertheless, it is still not clear what the physiological function of the A18 hnRNP protein is and whether DNA damage induction of the A18 hnRNP gene is p53 dependent. In addition, up to 13 DNA damage-inducible proteins were found to be capable of binding to a viral RNA probe consisting of the *trans*-activation-responsive element of human immunodeficiency virus type 1 and to a G+C-rich RNA probe (11). Since the genes encoding these RNA-binding proteins have not been characterized, it is not clear whether any of these genes can be regulated by p53.

How does the MCG10 protein mediate p53-dependent apoptosis and cell cycle arrest in G_2-M? Based on the activities conferred by the KH domain in other proteins, it is likely that MCG10 may regulate expression of genes responsible for the control of the cell cycle by both transcriptional and posttranscriptional mechanisms. For example, by binding to the CT-rich repeat elements in the promoter of c-*myc*, hnRNP K enhances transcriptional initiation, possibly by promoting remodeling of chromatin architecture to facilitate interactions between transcription factors (59, 60, 87). In contrast, by binding to the CT-rich element adjacent to the Sp1-responsive element (E2) in the promoter of the neuronal nicotinic acetylcholine receptor β4 subunit (nACH β4) gene, hnRNP K may directly block Sp1 binding to E2, leading to transcriptional repression of the nACH β4 gene (26). In addition, hnRNP K and E can bind to a CU-rich repetitive element in the 3′ untranslated region (3′-UTR) of erythroid 15-lipooxygenase (LOX) mRNA and block 80S ribosome complex assembly on LOX RNA, leading to translational silencing of the LOX gene (73). In contrast, by binding to a CU-rich RNA element in the 3′-UTR of α-globin mRNA, hnRNP E can stabilize α-globin mRNA, leading to enhanced expression of the α-globin gene (45, 97). Interestingly, five GADD mRNAs, including GADD45, which is a cellular target of p53 and whose product can mediate cell cycle arrest in G_2-M (95), are stabilized in hamster cells when treated with DNA-damaging agents (40). However, it is still not clear whether DNA damage-induced stabilization of these GADD mRNAs is p53 dependent. It will be interesting to determine whether MCG10 can regulate these GADD genes.

Tumorigenesis involves multistep sequential alterations of genetic materials. One of the early outcomes of this process is immortalization of cells, leading to an unlimited replicative life span. Recent studies have shown that overexpression of telomerase, whose activity can be regulated by p53 (16, 49), immortalizes cells, suggesting that the length of the telomere is critical for a limited replicative life span (19). Telomerase is a specialized reverse transcriptase that synthesizes a DNA sequence using an RNA template (19, 54). The RNA template is usually 100 to 200 nucleotides long and contains several repeats of C-rich elements. Interestingly, loss of heterozygosity (LOH) at 3p21, the mapped location of *MCG10*, is associated with an increased telomerase activity in head, neck, and renal carcinomas (55, 58). Since MCG10 is a potent poly(C)-binding protein, it is possible that, by binding to the C-rich repeats in the RNA template, MCG10 and MCG10as can sequester the RNA template and inhibit telomere synthesis, thereby suppressing cell proliferation.

In addition to the RNA-binding motifs, hnRNPs often contain other auxiliary domains, most notably the proline-rich PXXP motif (P represents proline, whereas X is any amino acid). PXXP residues can form a left-handed polyproline type II helix, which creates a binding site for Src homology 3 (SH3) domains (18). The proline-rich domains in hnRNP K and Sam68 have been shown to interact with several protooncogene products, including Src (85, 98), Fyn (98), Lyn (98), and Vav (10, 36). In addition, upon interaction with Src, hnRNP K and Sam68 can be phosphorylated at tyrosine residues by Src tyrosine kinase (85, 89). These results support a hypothesis that extracellular signals can be received by a membrane-associated tyrosine kinase, such as Src, which transmits the signal to an

RNA-binding protein, such as hnRNP K and Sam68. The RNA-binding protein would then regulate the expression of genes that control cellular responses to various extracellular signals. MCG10 and MCG10as contain three proline-rich domains at their carboxyl termini. Therefore, future studies are needed to determine with what protein MCG10 and MCG10as interact and what the physiological response is, if indeed an interaction occurs.

Most p53 target genes can mediate one defined p53 activity. For example, p21 is necessary for mediating G_1 arrest (7, 20), 14-3-3σ mediates G_2-M arrest (35), and Bax possibly mediates apoptosis (62). Interestingly, MCG10 and MCG10as can mediate two p53 activities, that is, apoptosis and cell cycle arrest in G_2-M. This may not be surprising. Since the mechanism by which MCG10 and MCG10as may function as a potential p53 mediator is their ability to regulate gene expression and/or to interact with one or more signaling proteins responsible for the control of the cell cycle, multiple pathways could be regulated. It should be noted that MCG10 and MCG10as are potent in inducing apoptosis, but unlike wild-type p53, they do so without inducing significant cellular DNA fragmentation. Since the RNA-binding activity is necessary for apoptosis, it is likely that one or more cellular genes whose products can lead to DNA breakdown are not regulated by MCG10 and MCG10as. Indeed, caspase 3 is not significantly activated by MCG10 (Fig. 6B). Caspase 3 is the primary effector enzyme that proteolytically inactivates DFF45 (DNA fragmentation factor 45) (also called ICAD [inhibitor of caspase-activated DNase]) and releases active DFF40 (also called CAD [caspase-activated DNase]), leading to internucleosomal DNA cleavage (102).

Is MCG10 a tumor suppressor? p53 is a bona fide tumor suppressor because it fulfills the "classical features" of a tumor suppressor (17). The ability of MCG10 to inhibit the growth of transformed cells fulfills one of the criteria for a tumor suppressor. Second, the *MCG10* gene maps to chromosome 3p21, a region highly susceptible to aberrant chromosomal rearrangements and deletions (61). LOH at 3p21 has been found in many types of human cancers, such as breast carcinomas, small and non-small cell lung carcinomas, uterine and cervical carcinomas, renal cell carcinomas, head, neck, and oral squamous cell carcinomas, ovarian cancers, and pancreatic islet cell tumors (6, 24, 25, 31, 70, 72, 75, 100, 101). Homozygous deletions of 3p21 are also found in several lung tumors and lung cancer cell lines (86). In esophageal carcinomas, LOH at 3p21 is an early event, preceding loss of RB and p53 functions (63). In addition, LOH in a region syntenic with 3p21 is also found in many types of mouse cancers (22, 70). When *scid* mouse tumors, which are induced by human chromosome 3-mouse microcell hybrids, were used to screen for a common eliminated region, one was often found at 3p21 (38, 44), suggesting that loss of a tumor suppressor gene may be necessary for microcell hybrids to induce tumors in *scid* mice. The human mismatch repair gene (hMLH) also maps to 3p21, and loss of hMLH function is associated with microsatellite instability at one or more loci (51). However, only a subset (less than 30%) of non-small cell lung carcinomas contain LOH at 3p21 with microsatellite instability (99), suggesting that, in non-small cell lung carcinomas without microsatellite instability, LOH at 3p21 probably involves another tumor suppressor gene(s). Therefore, future studies are needed to determine whether MCG10 LOH occurs in these tumors and whether loss of MCG10 contributes to tumorigenesis.

ACKNOWLEDGMENTS

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Appendix 7

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SHORT REPORT

Dickkopf-1, an inhibitor of the Wnt signaling pathway, is induced by p53

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Dickkopf-1 (Dkk-1), a secreted glycoprotein, has been found to be necessary and sufficient for inducing amphibian head formation. Interestingly, the mechanism by which Dkk-1 does this is the ability of Dkk-1 to antagonize the Wnt signaling pathway. Wnt, itself a proto-oncoprotein, can promote cell proliferation and transformation when mutated or overexpressed, leading to tumor formation. p53 is a tumor suppressor and loss of p53 function accelerates mammary tumorigenesis by Wnt. In this study, we found that Dkk-1 is induced by wild-type p53 but not mutant p53(R249S). In addition, DNA damage upregulates Dkk-1 in cell lines that harbor an endogenous wild-type p53 gene but not in cell lines that are p53-null or harbor an endogenous mutant p53 gene. We also found a potential p53 responsive element located approximately 2100 nucleotides upstream of the Dkk-1 transcription start site and we show that p53 binds specifically to this element both *in vitro* and *in vivo*. Furthermore, we have established several cell lines derived from H1299 lung carcinoma and U118 glioma cells that inducibly express Dkk-1 under a tetracycline-regulated promoter. We found that Dkk-1 has no effect on proliferation of cells that are not transformed by Wnt. Taken together, these results suggest that Dkk-1 may mediate p53 tumor suppression by antagonizing the Wnt signaling pathway. *Oncogene* (2000) **19,** 1843–1848.

Keywords: p53; Dickkopf-1; Wnt

The Wnt genes, encoding a large family of secreted, cysteine-rich glycosylated proteins, are evolutionarily conserved among diverse organisms such as *Homo sapiens*, *Mus musculus*, *Xenopus laevis*, *Drosophila melanogaster*, and *Caenorhabditis elegans* (Brown and Moon, 1998; Nusse and Varmus, 1992; Wodarz and Nusse, 1998). Genetic studies have demonstrated that the Wnt proteins serve as intercellular signaling molecules and play key roles in embryogenesis, segment polarity, central nervous system (CNS) patterning, and the control of asymmetric cell divisions (Wodarz and Nusse, 1998). Wnt signaling events are initiated by the binding of Wnt to its receptor, Frizzled (Krasnow *et al.*, 1995; Wong and Adler, 1993), which leads to activation of the Dishevelled protein (Klingensmith *et al.*, 1994; Krasnow *et al.*, 1995; Theisen *et al.*, 1994). The activated Dishevelled protein enhances the phosphorylation of glycogen synthase kinase (GSK) (Cook *et al.*, 1996), which inhibits the ability of GSK to phosphorylate β-catenin, leading to increased stability and accumulation of β-catenin (Munemitsu *et al.*, 1996; Pai *et al.*, 1997; Yost *et al.*, 1996). β-catenin can interact with members of T cell factor (TCF)/lymphoid enhancer factor (LCF) family in the nucleus, which regulates Wnt target genes necessary for development (Wodarz and Nusse, 1998).

Abnormal activation of the Wnt signaling pathway can lead to developmental catastrophe, such as duplication of the embryonic axis and subsequent induction of two-headed embryos in *Xenopus laevis*, and tumor formation in the mouse and human (Brown and Moon, 1998; Nusse and Varmus, 1992; Wodarz and Nusse, 1998). Recent studies in *Xenopus* embryos have identified at least four families of inhibitors of the Wnt signaling pathway, that is, Frizzled-related protein (FRP), Cerberus, Wnt-inhibitory factor-1 (WIF-1), and Dickkopf-1 (Dkk-1). Cerberus and WIF-1 physically interact with and inhibit Wnt (Glinka *et al.*, 1997; Hsieh *et al.*, 1999; Piccolo *et al.*, 1999). FRP inhibits the Wnt signaling pathway by physically associating with both Wnt and its receptor, Frizzled (Bafico *et al.*, 1999; Finch *et al.*, 1997; Leyns *et al.*, 1997; Wang *et al.*, 1997). Dkk-1 inhibits Wnt-mediated axis duplication in *Xenopus* (Glinka *et al.*, 1998). In addition, by inhibiting the Wnt signaling pathway, Dkk-1 is sufficient and necessary for head induction (Glinka *et al.*, 1998). Furthermore, Dkk-1 suppresses the ability of Wnt to promote cell proliferation (Fedi *et al.*, 1999). However, the mechanism by which Dkk-1 inhibits the Wnt signaling pathway and how Dkk-1 is regulated are still not clear.

p53 is a checkpoint protein. A large body of evidence suggests that p53 plays an important role in the regulation of numerous processes including cell cycle progression, differentiation, and apoptosis (Argarwal *et al.*, 1998; Almog and Rotter, 1997; Ko and Prives, 1996; Levine, 1997). Loss or mutation of p53 in some tumors has been correlated with a marked decrease of apoptosis and/or with a marked increase of cell proliferation. As a result, p53 deficiency can convert a slow growing tumor to a rapidly growing one (Howes *et al.*, 1994; Pan and Griep, 1995; Symonds *et al.*, 1994). Indeed, tumors appear at an earlier age in Wnt-1 and *ras* transgenic mice lacking p53 than in animals carrying one or both alleles of the p53 gene (Donehower *et al.*, 1995; Hundley *et al.*, 1997). Interestingly, the early onset of tumors in the Wnt-1 and *ras* transgenic mice is due to enhanced tumor cell proliferation but not decreased apoptosis in the absence of p53. Thus, p53 activities are necessary for inhibiting the acquired growth potential of tumor cells conferred by Wnt and *ras* (Hundley *et al.*, 1997; Jones *et al.*, 1997).

p53 transcriptional activity is necessary for tumor suppression (Chen, 1999; el-Deiry, 1998; Ko and Prives, 1996; Levine, 1997). p53 directly binds to DNA in a sequence specific manner and transactivates

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cellular target genes. A number of cellular genes has been found to be induced by p53. Among these are p21 (el-Deiry *et al.*, 1993), GADD45 (Kastan *et al.*, 1992), BAX (Miyashita *et al.*, 1994), MDM2 (Wu *et al.*, 1993), BTG2 (Rouault *et al.*, 1996), PIGs (Polyak *et al.*, 1997), 14-3-3σ (Hermeking *et al.*, 1997), IGFBP3 (Buckbinder *et al.*, 1995), PI(3)K regulatory subunit p85 (Yin *et al.*, 1998), KILLER/DR5 (Wu *et al.*, 1997), and TAP I (Zhu *et al.*, 1999). p21, 14-3-3σ, GADD45, and BTG2 have been shown to be capable of mediating p53-dependent cell cycle arrest (Chen *et al.*, 1996; el-Deiry *et al.*, 1993; Hermeking *et al.*, 1997; Rouault *et al.*, 1996; Wang *et al.*, 1999) while BAX, p85, IGFBP3, KILLER/DR5 and PIGs may mediate apoptosis (Buckbinder *et al.*, 1995; Miyashita *et al.*, 1994; Polyak *et al.*, 1997; Wu *et al.*, 1997; Yin *et al.*, 1998). Recently, we found that TAP1 is specifically induced by both p53 and p73, which leads to enhanced transport of MHC class I peptides, suggesting that tumor surveillance can be mediated by the p53 family tumor suppressor proteins (Zhu *et al.*, 1999).

In our ongoing effort to identify novel p53 target genes, the ClonTech PCR-Select cDNA Subtraction Assay was performed using mRNA isolated from p53-3, a derivative of the H1299 cell line that inducibly expresses p53 under a Tet-Off tetracycline-regulated promoter (Chen *et al.*, 1996). Several cDNA fragments that may represent gene activated by p53 were isolated. After DNA sequencing and comparison with known sequences in GenBank, one subtracted cDNA fragment was found to be derived from the Dkk-1 gene. To confirm that Dkk-1 is specifically induced by wild-type but not mutant p53, Northern blot analysis was performed using Dkk-1 cDNA as probe. We found that Dkk-1 was induced by p53 in p53-3 cells (Figure 1a, compare lanes 1 and 2). As a control, we tested expression of p21, a well-defined cellular p53 target gene (el-Deiry *et al.*, 1993). We found that p21 was also induced by p53 (Figure 1a, compare lanes 1 and 2). Furthermore, we found that mutant p53(R249S) was incapable of inducing either Dkk-1 or p21 (Figure 1a, compare lanes 3 and 4), consistent with the fact that

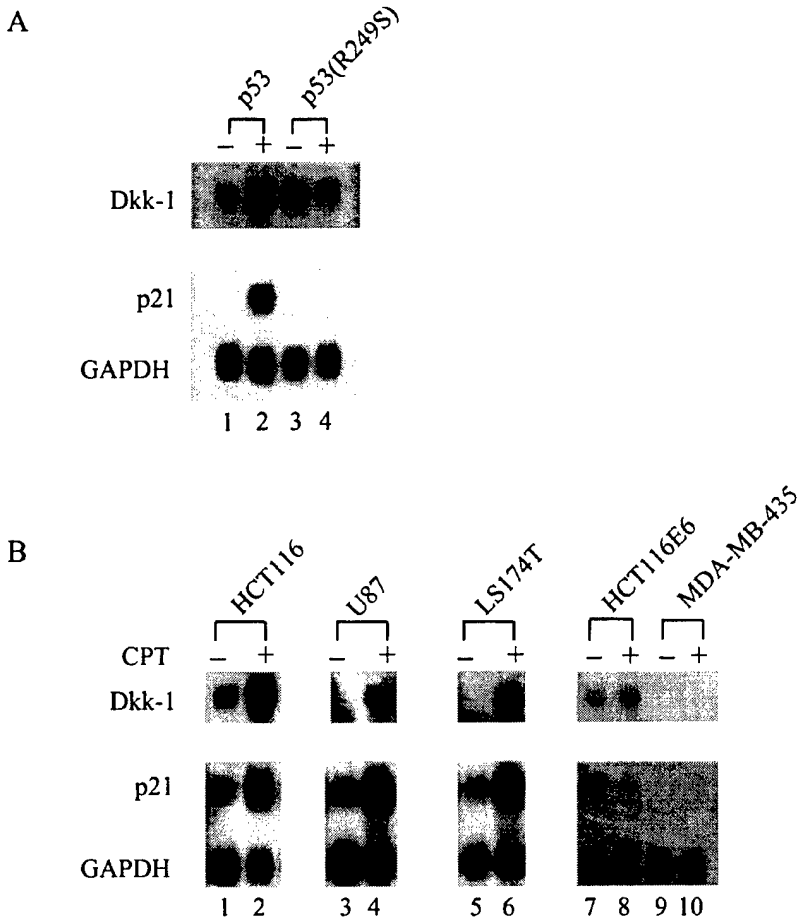

Figure 1 Upregulation of Dkk-1 by p53. (**a**) Wild-type p53, but not p53 mutant, induces Dkk-1. A Northern blot was prepared using 10 μg of total RNA isolated from p53-3 or p53(R249S)-4 cells that were uninduced (−) or induced (+) to express wild-type p53 and mutant p53(R249S), respectively. The blot was probed with Dkk-1 cDNA, and then reprobed with both p21 and GAPDH cDNAs. (**b**) Dkk-1 is induced by DNA damage in cell lines that carry an endogenous wild-type p53 gene but not in cells that are p53-null-like or contain an endogenous mutant p53 gene. Northern blots were prepared using 10 μg of total RNA isolated from HCT116, U87, LS174T, HCT116E6 or MDA-MB-435 cells that were untreated (−) or treated (+) with 300 nM camptothecin for 24 h. The blots were probed with Dkk-1 cDNA, and then reprobed with p21 and GAPDH cDNAs. Northern blot analysis was performed as described previously (Zhu *et al.*, 1998). p21 and GAPDH probes were prepared as described previously (Zhu *et al.*, 1998). Dkk-1 probe, a 600 bp *Hind*III fragment, was prepared from human Dkk-1 cDNA

this tumor-derived p53 mutant is defective in transactivation (Friedlander *et al.*, 1996). After normalization to the level of GAPDH mRNA, we estimated that the amount of Dkk-1 in cells expressing p53 was up to 6–8 times higher than in cells not expressing p53.

DNA damage stabilizes and activates p53, leading to induction of p53 target genes (Giaccia and Kastan, 1998; Ko and Prives, 1996; Levine, 1997). If Dkk-1 is a true p53 target, it would be induced by DNA damage in cells that contain an endogenous wild-type p53 gene but not in cell lines that are p53-null or contain an endogenous mutant p53 gene. To this end, we tested five cell lines using the DNA damaging agent camptothecin, which is an inhibitor of topoisomerase I and can induce double strand DNA breaks (Nelson and Kastan, 1994). These cells were treated with camptothecin and the levels of Dkk-1 and p21 determined by Northern blot analysis (Figure 1b). We found that both Dkk-1 and p21 were induced in camptothecin-treated HCT116, LS174T, and U87 cells,

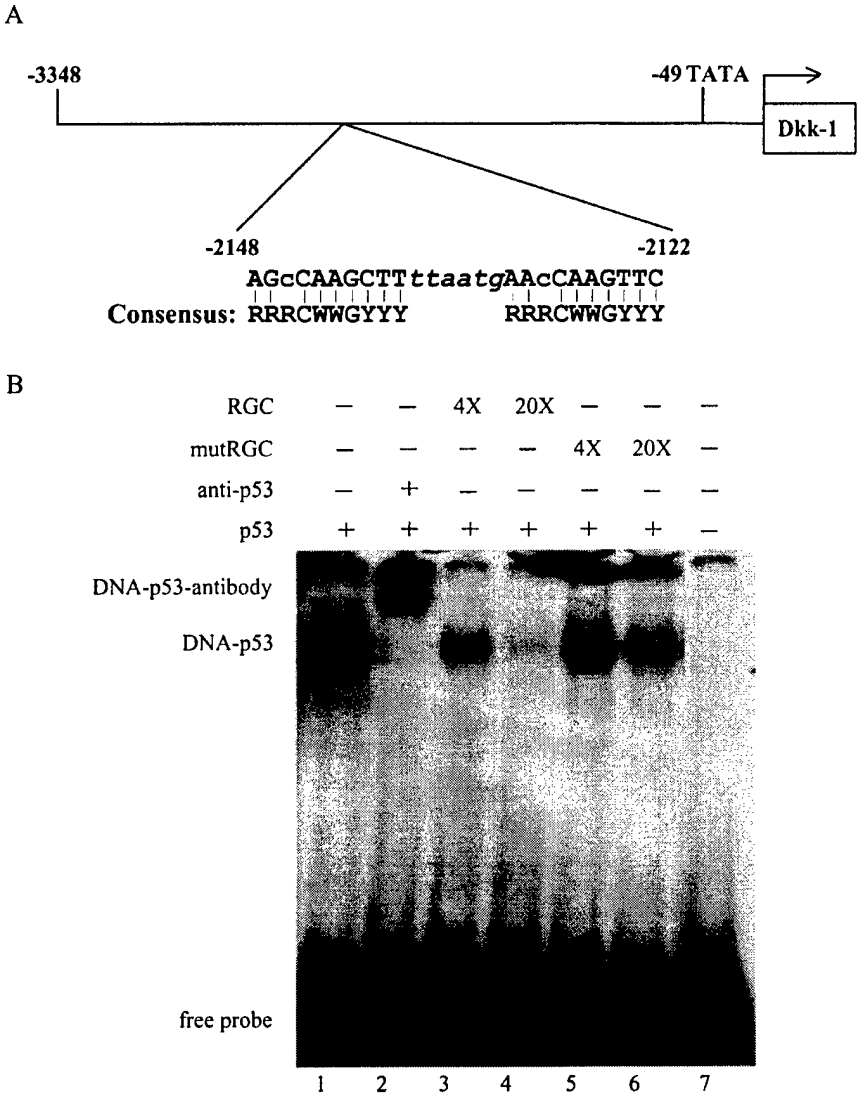

Figure 2 Identification of a p53-responsive element in the Dkk-1 gene. (**a**) Schematic representation of the Dkk-1 genomic DNA structure. The position of the potential Dkk-1 transcription start site and a potential p53 responsive element are indicated. Shown below the genomic structure are the sequence of the potential p53 responsive element and the previously characterized p53 consensus responsive element (el-Deiry *et al.*, 1992; Funk *et al.*, 1992). R represents purine, Y pyrimidine, and W adenine or thymidine. (**b**) p53 binds specifically to the potential p53 responsive element *in vitro*. A 32-bp oligonucleotide fragment containing the potential p53 responsive element in the Dkk-1 gene with the following sequence; 5′-AGCTTAGCCAAGCTTTTAATGAACCAAGTTCA-3′ (top strand) and 5′-GATCTGAACTTGGTTCATTAAAAGCTTGGCTA-3′ (bottom strand), was labeled with α-^{32}P-dCTP. 5 ng of the labeled probe DNA was added to a mixture [20 mM HEPES (pH 7.9), 25 mM KCl, 0.1 mM EDTA, 10% glycerol, 2 mM $MgCl_2$, 2 mM spermidine, 0.5 mM DTT, 0.025% NP-40, 100 ng double-stranded poly(dI:dC), and 2 μg BSA] containing 20 ng of p53 protein. The p53 protein was expressed in a baculovirus expression system and affinity-purified using anti-p53 monoclonal antibody Pab421. The p53-DNA complex was resolved in a 4% polyacrylamide gel. For 'supershifting' the p53-DNA complex, 1 μg of anti-p53 monoclonal antibody Pab1801 was added in the reaction in lane 2. For competition assays, unlabeled wild-type RGC (20 and 100 ng) or mutant RGC (20 and 100 ng) were added to the reaction run in lanes 3–4 and 5–6, respectively

which all contain wild-type p53 (Figure 1b, lanes 1–6). In contrast, Dkk-1 was not induced in p53-null-like HCT116E6 cells and MDA-MB-435 cells that carry an endogenous mutant p53 gene (Figure 1b, lanes 7–10). It should be noted that in HCT116 cells, the basal level of Dkk-1 expression is much higher than that in U87 and LS174T cells. Since several potential binding sites for other transcription factors in addition to p53 are present in the promoter region of the Dkk-1 gene (more discussion below), it is possible that one or more transcription factors may be responsible for the high basal level of Dkk-1 expression in HCT116 cells.

To determine whether Dkk-1 is regulated transcriptionally by p53, we searched for a p53-responsive element in the Dkk-1 genomic DNA sequence. Using Dkk-1 cDNA as probe, we screened a human bacterial artificial chromosome (BAC) library and obtained a genomic DNA clone that contains the human Dkk-1 gene. An approximately 3.4-kb DNA in the promoter region of the Dkk-1 gene was sequenced. We found a potential p53-binding site located approximately 2.1-kb upstream of the Dkk-1 transcription start site (Figure 2a). This sequence (AGc CAAG CTT TTAATG AAc CAAG TTC) has two mismatches (cytosine in lower case instead of guanine or adenine) in the non-critical positions within the consensus p53-binding site (el-Deiry *et al.*, 1992; Funk *et al.*, 1992).

To analyse whether p53 binds to the potential p53 responsive element, a 32-bp DNA fragment containing this element was synthesized, ^{32}P-labeled, and used in an electrophoretic mobility shift assay (EMSA). We found that when the purified p53 protein was mixed with the DNA fragment, a complex that presumably contained both p53 and DNA was detected (Figure 2b, lane 1). The complex was 'supershifted' with the anti-p53 monoclonal antibody Pab1801 (lane 2). We also used two other DNA fragments that contain either a wild-type or mutant p53-binding site from the ribosomal gene cluster (RGC) (Kern *et al.*, 1991) as competitors. The unlabeled wild-type RGC competed with the ^{32}P-labelled 32-bp DNA fragment from the Dkk-1 gene and inhibited the formation of the p53-DNA complex in a dose-dependent manner (lanes 3 and 4). In contrast, mutant RGC was unable to compete (lanes 5 and 6). These results indicate that p53 interacts specifically with the potential p53 responsive element in the Dkk-1 gene.

We further examined whether the potential p53-binding site is responsive to p53 *in vivo*. To do this, the potential p53 responsive element was cloned upstream of a minimal c-*fos* promoter (Johansen and Prywes, 1994) and a luciferase reporter gene to generate a reporter vector, designated Dkk-1-Fluc. We also substituted four nucleotides in the potential p53 responsive element predicted to be critical for p53-binding (shown in lower case) (AGC aAAt CTT T-TAATG AAC aAAt TTC). We then generated a reporter vector designated mut-Dkk-1-Fluc. Dkk-1-Fluc or mut-Dkk-1-Fluc was cotransfected into H1299 cells with either pcDNA3 control vector or a vector that expresses one of the following: wild-type p53, p53(V143A), p53(R175H), p53(R249S), and p53 (R273H). We found that the luciferase activity for Dkk-1-Fluc was markedly increased by wild-type p53 but not by any of the p53 mutants (Figure 3a). These results are consistent with the observation that wild-type p53, but not mutant p53(R249S), induces Dkk-1 (Figure 1a). In contrast, the luciferase activity for mut-Dkk-1-Fluc was not increased by either wild-type p53 or p53 mutants (Figure 3b).

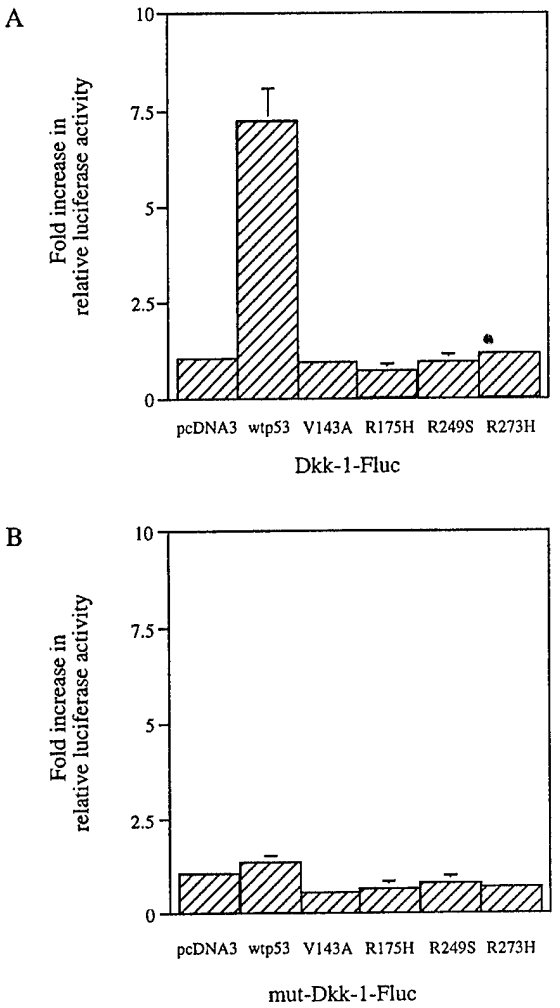

Figure 3 (a) The potential p53-binding site in the Dkk-1 gene is responsible to wild-type p53, but not p53 mutants *in vivo*. The 32-bp DNA fragment described in Figure 2 was cloned upstream of a minimal c-*fos* promoter and a firefly luciferase reporter gene (Johansen and Prywes, 1994), and the resulting construct designated Dkk-1-Fluc. Two μg of Dkk-1-Fluc was co-transfected into H1299 cells with 5 μg of pcDNA3 control vector or a vector that expresses wild-type p53, p53(V143A), p53(R175H), p53(R249S), or p53(R273H). Renilla luciferase assay vector pRL-CMV was also co-transfected as an internal control. Dual luciferase assay was performed according to the manufacturer's instruction (Promega, Madison, WI, USA). The fold increase in relative luciferase activity is a product of the luciferase activity induced by p53 divided by that induced by pcDNA3. (b) The mutated potential p53-binding site in the Dkk-1 gene is not responsive to either wild-type p53 or p53 mutants. A mutant version of the above 32-bp DNA fragment was made with the following sequence: 5′-AGCTTAGCaAAtCTTTTAATGAA-CaAAtTTCA-3′ (top strand) and 5′-GATCTGAAaTTtGTTCAT-TAAAAGaTTtGCTA-3′ (bottom strand). The substituted nucleotides are shown in lower case. The mutant fragment was then cloned upstream of a minimal c-*fos* promoter and a firefly luciferase reporter gene, and the resulting construct designated mut-Dkk-1-Fluc. Luciferase assays with mut-Dkk-1-Fluc were performed as in (a)

Activation of p53 leads to at least two well-characterized cellular responses: cell cycle arrest and apoptosis (Chen, 1999; Ko and Prives, 1996; Levine, 1997). We wanted to determine whether Dkk-1, as a cellular target of p53, mediates p53 tumor suppression. To test this possibility, Dkk-1 was inducibly expressed in H1299 lung carcinoma and U118 glioma cells under a Tet-Off-tetracycline-regulated promoter. H1299 is p53-null but U118 harbors a p53 gene that can be activated by DNA damage (data not shown). Western blots from representative H1299 cell lines and U118 cell lines showed that, when induced, Dkk-1 was expressed with an apparent molecular mass of 29–35 kDa (data not shown). This range is consistent with previous reports of a slower migrating form of Dkk-1 that is N-linked glycosylated (Fedi *et al.*, 1999; Glinka *et al.*, 1998). We then measured the growth rates of these cells in the absence or presence of Dkk-1. We found that Dkk-1 has little, if any, effect on the growth rates of either H1299 or U118 cells (data not shown). In addition, no cell cycle arrest and apoptosis were detected using DNA histogram analysis and annexin V staining assay (data not shown). Thus, while Dkk-1 can suppress Wnt-induced transformation (Fedi *et al.*, 1999), it has no effect on the proliferation of cells that are not transformed by Wnt.

In this report we have demonstrated that Dkk-1 can be induced by p53 and DNA damage. We found a p53 responsive element located approximately 2100 nucleotides upstream of the Dkk-1 transcription start site, which may mediate DNA damage induction of Dkk-1. We also found that Dkk-1 has no effect on proliferation of cells that are not transformed by Wnt. Nevertheless, previous studies have shown that Dkk-1 is a potent antagonist of Wnt signaling necessary and sufficient for head induction in *Xenopus* (Glinka *et al.*, 1998) and that human Dkk-1 strongly suppresses Wnt-induced morphological transformation (Fedi *et al.*, 1999). Biochemical and genetic studies have shown that Dkk-1 antagonizes the Wnt signaling pathway, upstream of β-catenin and Dishevelled (Fedi *et al.*, 1999; Glinka *et al.*, 1998). Taken together, we propose that, by inducing Dkk-1, p53 plays an important role in suppressing Wnt-mediated tumor formation. Therefore, p53 dysfunction would alleviate the negative control of Wnt signaling by Dkk-1. As a result, uncontrolled Wnt signaling may be responsible for the early onset of mammary tumors in p53-null Wnt transgenic mice (Donehower *et al.*, 1995; Jones *et al.*, 1997).

Although the Wnt genes were initially identified as candidate proto-oncogenes, ectopic expression of Wnt induces axis duplication in *Xenopus* and Wnt gene deficiency prevents normal development of CNS, placenta, limbs, kidney, caudal somites and tailbud (Brown and Moon, 1998; Nusse and Varmus, 1992; Wodarz and Nusse, 1998). The negative control of Wnt signaling by Dkk-1 is also necessary for normal development (Glinka *et al.*, 1998). Interestingly, p53 induces Dkk-1 but p53 activity is not necessary for normal development in mice (Donehower *et al.*, 1992). This suggests that other factor(s) must be responsible for proper expression of Dkk-1 during development. In addition to the p53 responsive element, several potential regulatory elements, such as Sp1, MyoD, STAT, Oct-1/2, C/EBP-α/β, and GATA-1, -2 and -3, are found in the promoter region of the Dkk-1 gene (data not shown). Determining whether these transcription factors regulate Dkk-1 would lead to further our understanding of the role of Dkk-1 in development.

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p53 induces TAP1 and enhances the transport of MHC class I peptides

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The transporter associated with antigen processing (TAP) 1 is required for the major histocompatibility complex (MHC) class I antigen presentation pathway, which plays a key role in host tumor surveillance. Since more than 50% of tumors have a dysfunctional p53, evasion of tumor surveillance by tumor cells may be linked to loss of p53 function. Here we found that TAP1 is strongly induced by p53 and DNA-damaging agents through a p53-responsive element. We also found that p73, which is homologous to p53, is capable of inducing TAP1 and cooperates with p53 to activate TAP1. Furthermore, we found that by inducing TAP1, p53 enhances the transport of MHC class I peptides and expression of surface MHC-peptide complexes, and cooperates with interferon γ to activate the MHC class I pathway. These results suggest that tumor surveillance may be a mechanism by which p53 and/or p73 function as tumor suppressors.

Keywords: p53; TAP1; MHC class I; interferon γ; tumor surveillance

Introduction

p53 is one of the most frequently mutated genes in cancer. More than 50% of all human tumors contain a dysfunctional p53 (Hollstein *et al.*, 1991). It is well established that p53 plays an important role in the regulation of cell cycle, apoptosis, differentiation, and in the maintenance of genome integrity (Chen, 1999; Almog and Rotter, 1998; Ko and Prives, 1996; Levine, 1997), all of which contribute to p53 tumor suppression. As a sequence-specific transcription factor, p53 up-regulates expression of several cellular genes, for example, p21 and 14-3-3σ that mediate p53-dependent cell cycle arrest (el-Deiry *et al.*, 1993; Hermeking, 1997), and BAX and a group of redox-related genes (PIGs) that may mediate p53-dependent apoptosis (Miyashita *et al.*, 1994; Polyak *et al.*, 1997).

p53 is a multifunctional protein. Mechanisms other than cell cycle arrest and apoptosis may also be involved in p53 tumor suppression. When normal cells become malignant, cellular proteins that are normally present at low levels may become over-expressed or the genes that encode these cellular proteins may become mutated, resulting in the production of tumor antigens (Old and Chen, 1998). These tumor antigens would then be processed and presented by the host major histocompatibility complex (MHC) class I antigen presentation pathway on the cell surface. Several proteins are necessary for the MHC class I pathway, including large multifunctional proteasome subunits 2 and 7 (LMP2 and LMP7), transporters associated with antigen processing 1 and 2 (TAP1 and TAP2), and two polypeptides for the MHC class I molecule, heavy chain HLA-ABC and light chain β_2 microglobulin (β_2M) (Pamer and Cresswell, 1998). LMP2 and LMP7 are involved in breaking down intracellular proteins into antigenic peptides. TAP1 and TAP2 are involved in the transport of these antigenic peptides from cytosol to endoplasmic reticulum where they bind to the assembled MHC class I molecules. The MHC-peptide complex is then transported to and expressed on the cell surface. Cytotoxic T lymphocytes (CTLs) recognize and attack cells with tumor antigens on the cell surface via an interaction between the T cell receptor and the MHC-peptide complex. However, during tumorigenesis, tumor cells acquire mutations that help them evade recognition by the immune system. One mechanism that tumor cells could use is to down-regulate the MHC class I pathway (Pamer and Cresswell, 1998; Restifo *et al.*, 1993b). Without stable MHC-peptide complexes on the cell surfaces, tumor cells evade CTL recognition.

As part of our ongoing effort to understand p53 function in cells, we used the ClonTech PCR-Select cDNA Subtraction assay to identify novel cellular p53 target genes. We found that TAP1 is specifically induced by both p53 and p73, which leads to enhanced transport of MHC class I peptides. These findings suggest that tumor surveillance can be mediated by the p53 family tumor suppressor proteins.

Results

Upregulation of TAP1 by p53

In an effort to identify new p53 target genes, the ClonTech PCR-Select cDNA Subtraction assay was performed using mRNA isolated from p53-3, a derivative of H1299 cell line that inducibly expresses p53 under a tetracycline-regulated promoter (Chen *et al.*, 1996b). Several cDNA fragments that may represent genes activated by p53 were isolated. After DNA sequencing, one subtracted cDNA fragment was found to be derived from the TAP1 gene. To confirm that TAP1 can be induced by p53, Northern blot analysis was performed using TAP1 cDNA as probe. We found that TAP1 was induced in p53-3 cells when p53 was expressed (Figure 1a, compare lanes 1 and 2). As a control, we tested expression of p21, a well-defined cellular p53 target gene (el-Deiry *et al.*, 1993).

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We found that p21 was also induced by p53 (Figure 1a, compare lanes 1 and 2). Furthermore, we found that mutant p53(R249S) was incapable of activating both TAP1 and p21 (Figure 1a, compare lanes 3 and 4), consistent with the fact that this tumor-derived p53 mutant is defective in transactivation. After normalization to the level of GAPDH mRNA, we estimated that the amount of TAP1 in cells expressing p53 was 4–6 times higher than in cells not expressing p53.

Since the p53 protein is stabilized and accumulates in cells following DNA damage (Ko and Prives, 1996), we determined whether TAP1 can be activated by DNA damage in the RKO colorectal carcinoma cell line, which contains an endogenous wild-type p53 gene (Nelson and Kastan, 1994). To this end, RKO cells were treated with camptothecin, doxorubicin, or actinomycin D. Camptothecin and doxorubicin are inhibitors of topoisomerase I and II, respectively, both of which induce double-strand DNA breaks (Nelson and Kastan, 1994). Actinomycin D inhibits transcription, but induces DNA damage at low concentrations (1–10 nM) (Nelson and Kastan, 1994). Northern blot analysis showed that TAP1 was induced in RKO cells treated with these DNA-damaging agents (Figure 1b). As expected, p21 was also activated (Figure 1b). After normalization to the level of GAPDH mRNA, we found that the amount of TAP1 expressed in RKO cells treated with these DNA-damaging agents was 4–8 times greater than in mock-treated cells.

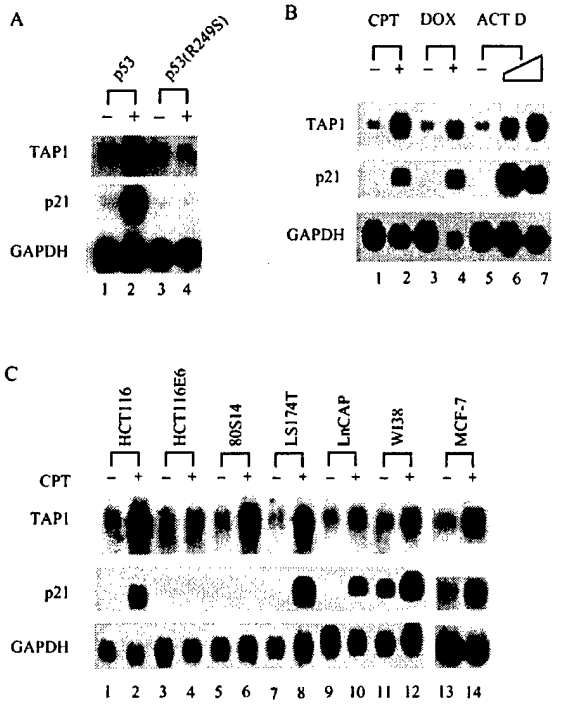

Figure 1 (a) Wild-type p53, but not p53 mutant, induces TAP1. A Northern blot was prepared using 10 μg of total RNA isolated from p53-3 or p53(R249S)-2 cells that were uninduced (−) or induced (+) to express wild-type p53 and mutant p53(R249S), respectively. (b) TAP1 is induced by three DNA-damaging agents in RKO cells. A Northern blot was prepared using 10 μg of total RNA isolated from untreated RKO cells (−) or cells treated (+) with 300 nM camptothecin (CPT), 1.0 μg/ml doxorubicin (DOX), 3.0 or 10 nM actinomycin D (ACT D). (c) TAP1 is induced by DNA damage in six cell lines that carry an endogenous wild-type p53 gene but not in one that is functionally p53-null. Northern blots were prepared using 10 μg of total RNA isolated from seven individual cell lines as indicated at the top of the figure, which were untreated (−) or treated (+) with 300 nM camptothecin for 24 h. The blots were probed with TAP1 cDNA, and then reprobed with p21 and GAPDH cDNAs, respectively

If TAP1 is a true cellular p53 target, TAP1 should be induced by p53 (i.e., DNA damage) in other cell lines that contain an endogenous wild-type p53 gene but not in cell lines that are p53-null. To this end, we tested seven different cell lines. HCT116, LS174T, LnCap, WI-38, and MCF7 each contain an endogenous wild-type p53 gene. 80S14 cell line is an HCT116 derivative that is p21-null (Waldman *et al.*, 1996), and HCT116E6 is an HCT116 derivative that contains human papillomavirus (HPV) oncoprotein E6. Since HPV E6 facilitates degradation of p53 (Ko and Prives, 1996), HCT116E6 is a p53-null-like cell line. These cells were treated with camptothecin and the levels of TAP1 and p21 determined by Northern blot analysis (Figure 1c). We found that both TAP1 and p21 were induced in cells containing wild-type p53 when treated with camptothecin (Figure 1c, lanes 1,2 and 7–14). Although p21 was not expressed in the p21-null 80S14 cells, TAP1 was still induced by DNA damage (Figure 1c, lanes 5 and 6), indicating that p53 can activate TAP1 independently of p21. In contrast, TAP1 was not induced in p53-null-like HCT116E6 cells (Figure 1c, lanes 3 and 4).

Since p73 is homologous to p53 (Kaghad *et al.*, 1997) and is capable of inducing p21 (Jost *et al.*, 1997; Kaghad *et al.*, 1997; Zhu *et al.*, 1998a), we wanted to determine whether TAP1 is a common cellular target of p53 and p73. To this end, we used three H1299 cell lines that inducibly express two alternatively spliced forms of wild-type p73, i.e., p73α and p73β, and one mutant p73α292, respectively (Zhu *et al.*, 1998a). We found that both TAP1 and p21 were induced by both wild-type p73α and p73β but not by mutant p73α292 (Figure 2a). Since both p53 and p73 are activators of transcription, they may cooperate to activate genes responsible for tumor suppression. To determine whether TAP1 is activated cooperatively by p53 and p73, TAP1 expression was examined in MCF7 cells that are either induced to express p73α, treated with camptothecin to induce p53, or both induced to express p73α and treated with camptothecin to induce p53 (Figure 2b). We found that TAP1 was up-regulated in MCF7 cells when treated with camptothecin (Figure 2b, compare lanes 1 and 2) or induced to express p73α (Figure 2b, compare lanes 1 and 4). After PhosphorImage quantitation, we found that TAP1 was induced 2.6-fold by either p53 or p73α. In contrast, TAP1 was induced 7.1-fold when both p53 and p73α were expressed in MCF7 cells (Figure 2b, lane 3). These results suggest that p73α and p53 (DNA damage) cooperate to activate TAP1 expression. We also found that p21 was activated cooperatively by p73α and DNA damage-induced p53 in MCF7 cells (Figure 2b).

Because TAP1 is one of the components required for the MHC class I antigen presentation pathway, we wanted to determine whether other genes in this pathway are regulated by p53. We examined five other genes by Northern blot analysis and found that TAP2, LMP2, LMP7 and MHC class I heavy chain

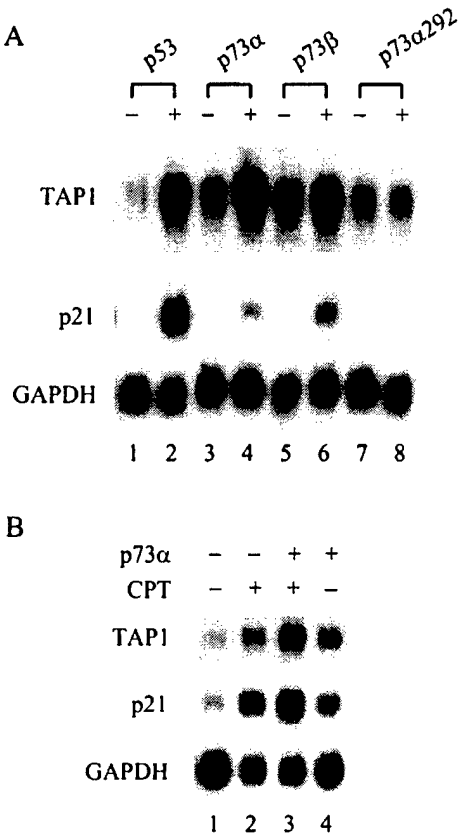

Figure 2 (**a**) Wild-type p73, but not mutant p73, is capable of inducing TAP1. A Northern blot was prepared using 10 μg of total RNA isolated from uninduced cells (lanes 1, 3, 5 and 7) or cells that were induced to express wild-type p53 (lane 2), p73α (lane 4), p73β (lane 6), or mutant p73α292 (lane 8). (**b**) p73 cooperates with DNA damage to activate TAP1 in MCF7 cells that carry an endogenous wild-type p53 gene. A Northern blot was prepared using 10 μg of total RNA isolated from MCF7 cells that were untreated (lane 1), treated with 300 nM camptothecin (CPT) to induce endogenous wild-type p53 (lane 2), induced to express exogenous p73α and treated with 300 nM camptothecin to induce endogenous wild-type p53 (lane 3), or induced to express exogenous p73α (lane 4). The blots were probed with TAP1, p21, and GAPDH cDNAs, respectively

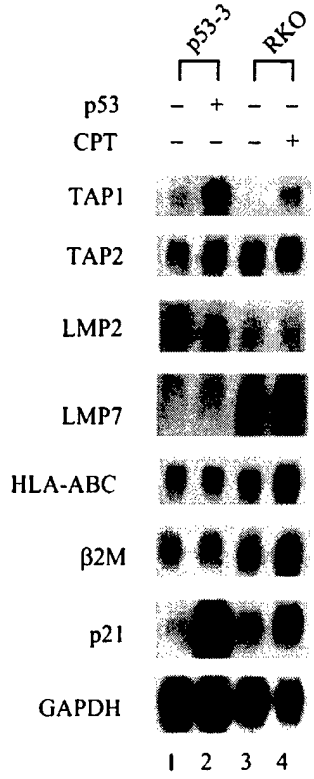

Figure 3 TAP1, but not other components in the MHC class I pathway, is induced by p53. Northern blots were prepared using 10 μg of total RNA isolated from p53-3 cells that were uninduced (–) or induced (+) to express exogenous wild-type p53, or from RKO cells that were untreated (–) or treated (+) with 300 nM camptothecin to induce endogenous wild-type p53 for 24 h. The blots were probed with TAP1, TAP2, LMP2, LMP7, HLA-ABC, β_2M, p21 and GAPDH cDNAs, respectively

HLA-ABC and light chain β_2M were expressed, but not significantly induced by p53 or DNA damage in p53-3 and RKO cells, respectively (Figure 3).

Next, we examined the level of TAP1 protein in p53-3 cells by Western blot analysis. We found that p53 expression resulted in the increase of TAP1 protein (Figure 4, compare lanes 1 and 2), consistent with p53 induction of TAP1 mRNA as analysed by Northern blot analysis (Figure 1a). p53-3 cells were also treated with 5, 15, 50, 100 and 500 U of IFNγ, a potent inducer of TAP1 (Stark *et al.*, 1998). We found that the TAP1 protein was efficiently induced with 15 U/ml of IFNγ (Figure 4, lane 3).

Identification of a specific p53-responsive element in the TAP1 gene

To define whether TAP1 is a true target of p53, we searched for a p53-responsive element in the genomic DNA sequence of the TAP1 gene. A potential p53-

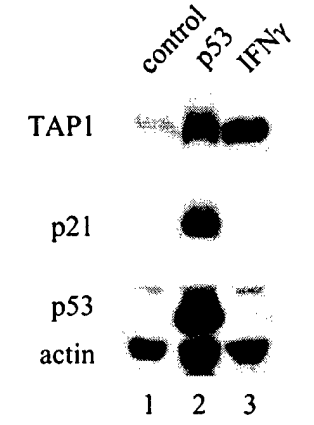

Figure 4 The TAP1 protein is increased in cells expressing p53 or treated with IFNγ. The levels of TAP1, p21, p53, and actin proteins in p53-3 cells that were untreated (lane 1), induced to express p53 (lane 2), or treated with 15 U/ml of IFNγ (lane 3), were assayed by Western blot analysis. The blots were probed with anti-TAP1 monoclonal antibody A148.3, anti-p21 monoclonal antibody, and a mixture of anti-p53 monoclonal antibody Pab1801 and anti-actin polyclonal antibody, respectively

binding site was found to be located approximately 300 nucleotides downstream of the TAP1 transcription start site (Beck *et al.*, 1992). This sequence (ggg cttg g*cc ctgccg gga cttg cct) has only one mismatch (G* instead of C/T) to the consensus p53-binding site (el-Deiry *et al.*, 1992). To analyse whether p53 binds to this sequence, a 59-bp DNA fragment containing this region was synthesized, ^{32}P-labeled, and used in an electrophoretic mobility shift assay (EMSA). We found that p53 interacts specifically with the potential p53-responsive element in the TAP1 gene (data not shown).

We further examined whether the potential p53-binding site is responsive to p53 *in vivo*. To do this, the potential p53-responsive element was cloned upstream of a minimal promoter and a luciferase reporter gene to generate the reporter vector TAP1-Fluc. The construct GADD45-Fluc, which contains a p53-responsive element from the GADD45 gene, a well-defined cellular p53 target, was used as a positive control as described previously (Chen *et al.*, 1995). We found that the luciferase activity for either TAP1-Fluc or GADD45-Fluc was markedly increased by wild-type p53 (Figure 5a), suggesting that p53 can bind to the p53-responsive elements from both the TAP1 and GADD45 genes. Interestingly, we observed that the increase in the luciferase activity by p53 for TAP1-Fluc was about five times greater than that for GADD45-Fluc (Figure 5a). This suggests that the p53-binding site in the TAP1 gene may have a higher affinity for p53 than the binding site in the GADD45 gene. Similarly, we found that the luciferase activity for TAP1-Fluc was increased by both p73α and p73β (Figure 5b). In contrast, the luciferase activity for TAP1-Fluc was not increased by the mutants p53(V143A), p53(R175H), p53(R249S), or p53(R273H) (Figure 5c), consistent with the observation that mutant p53(R249S) was incapable of inducing TAP1 (Figure 1a).

p53 induction of TAP1 leads to increased transport of MHC class I peptides

To determine whether induction of TAP1 by p53 can lead to increased transport of MHC class I peptides,

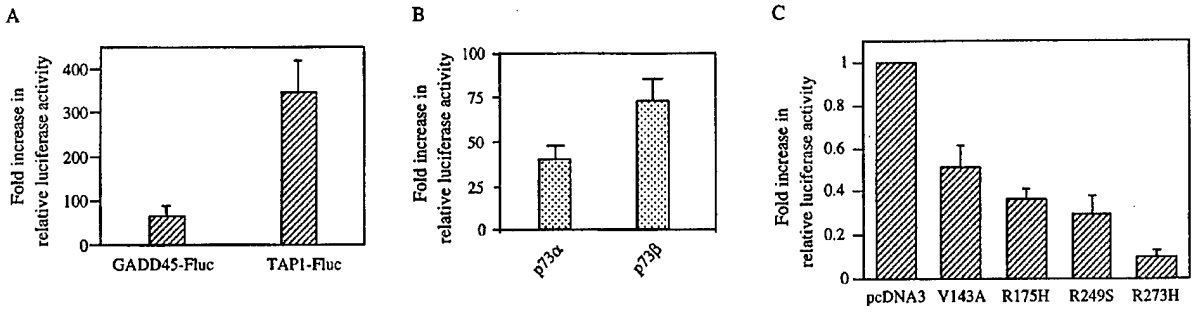

Figure 5 Wild-type p53 and p73 bind to the p53-responsive element *in vivo*. (**a**) The potential p53-binding site in the TAP1 gene is responsive to wild-type p53 *in vivo*. 5 μg of TAP1-Fluc or GADD45-Fluc was co-transfected into H1299 cells with 5 μg of pcDNA3 or a vector that expresses wild-type p53. The fold increase in relative luciferase activity is a product of the luciferase activity activated by p53 divided by that activated by pcDNA3. (**b**) The potential p53-binding site in the TAP1 gene is responsive to wild-type p73 *in vivo*. 5 μg of TAP1-Fluc was co-transfected into H1299 cells with 5 μg of pcDNA3 or a vector that expresses wild-type p73α or p73β. The fold increase in relative luciferase activity is calculated similarly to that in (**a**). (**c**) p53 mutants are unable to increase the luciferase activity for TAP1-Fluc. 5 μg of TAP1-Fluc was co-transfected into H1299 cells with 5 μg of pcDNA3 or a vector that expresses p53(V143A), p53(R175H), p53(R249S), or p53(R273H). The fold increase in relative luciferase activity was determined similarly to that in (**a**)

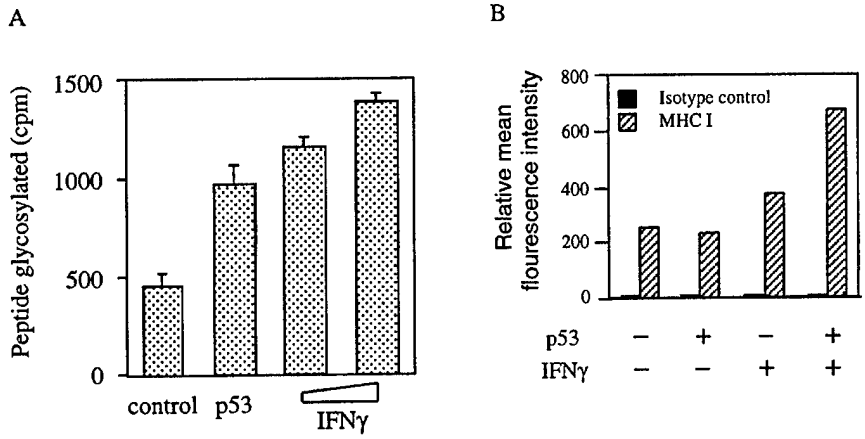

Figure 6 (**a**) p53 and IFNγ increases peptide transport capacity in p53-3 cells. p53-3 cells were uninduced, induced to express p53, or treated with 5 or 20 U/ml of IFNγ for 24 h. The extent of peptide glycosylation was then used to measure the relative peptide transport capacity in cells. (**b**) p53 cooperates with IFNγ to enhance the expression of surface MHC-peptide complexes. p53-3 cells that were uninduced or induced to express p53 were mock-treated or treated with 500 U/ml IFNγ for 48 h. The level of surface MHC-peptide complexes was determined by FACS analysis with anti-human HLA-ABC antibody B-H9. Mouse IgG1 was used as an isotype control

we performed peptide transport assays (Ma *et al.*, 1997). We found that the amount of glycosylated B27 peptide, a variant of an HLA-B27-binding, human histone 3 peptide, was significantly increased in p53-3 cells by p53 and IFNγ (Figure 6a). Similar results were obtained with A3 peptide, a variant of an HLA-A3-binding, HIV nef 7B peptide (data not shown). It should be noted that since IFNγ can also induce TAP2, the other key component for the transport of MHC class I peptides (Pamer and Cresswell, 1998), it is not surprising that IFNγ was more potent than p53 in enhancing the transport of B27 peptide (Figure 6a).

As MHC class I peptides are transported into the endoplasmic reticulum, they bind to assembled MHC class I molecules to form stable MHC-peptide complexes, which are subsequently expressed on the cell surface (Pamer and Cresswell, 1998). To determine whether p53 can increase the expression of surface MHC-peptide complexes on p53-3 cells, FACS analysis was performed. We found that the level of surface MHC-peptide complexes was not significantly increased by p53 (Figure 6b). This is not surprising since other abnormalities in the MHC class I pathway can inhibit MHC class I expression (Proffitt and Blair, 1997; Restifo *et al.*, 1993a). Indeed, the LMP7 gene, whose product is required for the generation of MHC class I peptides, was found to be expressed at an extremely low level in p53-3 cells (Figure 3). Consequently, the supply of cellular MHC class I peptides may be limited, which hinders the formation of stable MHC-peptide complexes. Therefore, we examined whether p53 can further increase MHC class I expression when p53-3 cells are treated with IFNγ to induce LMP7. We found that the level of MHC-peptides complexes expressed on IFNγ-treated cells was about 1.5 times higher than on untreated cells or cells expressing p53 (Figure 6b). However, when cells were both induced to express p53 and treated with IFNγ, the level of surface MHC-peptide complexes was 2.6 times greater than on untreated cells or cells expressing p53 alone (Figure 6b).

Since the LMP7 gene is highly expressed in the RKO cell line (Figure 3, LMP7 panel), we chose it to further determine whether p53 can enhance the transport of MHC class I peptides and expression of surface MHC-peptide complexes. As expected, when RKO cells were treated with camptothecin, the p53 protein was stabilized (Figure 7a, p53 panel), and subsequently, the TAP1 mRNA (Figure 7b, TAP1 panel) and protein (Figure 7a, TAP1 panel) upregulated. When RKO cells were treated with IFNγ, p53 was not stabilized (Figure 7a, p53 panel), but the TAP1 mRNA (Figure 7b, TAP1 panel) and protein (Figure 7a, TAP1 panel) were increased, suggesting that IFNγ can regulate the MHC class I pathway independently of p53 in RKO cells. To determine whether p53 is necessary for the enhanced expression of TAP1, we generated a derivative of the RKO cell line, RE6-26, which stably expresses HPV E6 oncoprotein. As a result, RE6-26 becomes a p53-null-like cell line. Indeed, p53 was undetectable in RE6-26 cells when treated with camptothecin (Figure 7a, compare lanes 4 and 5, p53 panel) and subsequently, the TAP1 mRNA (Figure 7b, TAP1 panel) and protein (Figure 7a, TAP1 panel) not induced. However, TAP1 was still induced in RE6-26 cells by IFNγ (Figure 7a, TAP1 panel; Figure 7b, TAP1 panel), suggesting that the IFNγ-regulated MHC class I pathway is not affected by the HPV E6 oncoprotein.

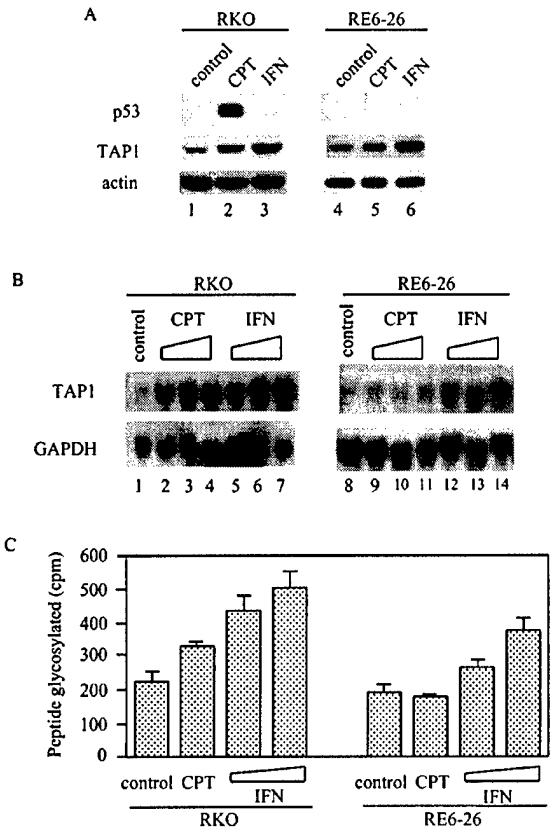

Figure 7 (a) p53 is required for the increased expression of TAP1 protein in RKO cells by DNA damage. The levels of TAP1, p53, and actin proteins in RKO and RE6-26 cells that were untreated (lanes 1 and 4), treated with 100 nM camptothecin (lanes 2 and 5), or 20 U/ml IFNγ (lanes 3 and 6), were assayed by Western blot analysis. The blots were probed with anti-p53 Pab1801, anti-TAP1 A148.3, and anti-actin polyclonal antibody, respectively. (b) p53 is required for the increased expression of TAP1 mRNA in RKO cells by DNA damage. Northern blots were prepared using 10 μg of total RNA isolated from RKO or RE6-26 cells that were untreated (lanes 1 and 8), treated with 50, 100, or 200 nM camptothecin (lanes 2–4 and 9–11), or treated with 10, 20, or 40 U/ml IFNγ (lanes 5–7 and 12–14) for 24 h. The blots were probed with TAP1 cDNA, and then reprobed with GAPDH cDNA. (c) p53 is required for the increased peptide transport capacity in RKO cells by DNA damage. Peptide transport assay was performed using RKO or RE6-26 cells that were untreated, treated with 100 nM camptothecin, or treated with 5 or 20 U/ml IFNγ for 24 h. The extent of peptide glycosylation was then used to measure the relative peptide transport capacity in cells

Next, we determined the peptide transport capacity in RKO and RE6-26 cells when treated with camptothecin or IFNγ. We found that the amount of glycosylated peptide was increased in RKO cells by both camptothecin and IFNγ (Figure 7c). In contrast, IFNγ, but not camptothecin, was capable of increasing the transport of MHC class I peptides in RE6-26 cells (Figure 7c).

To determine whether DNA damage can increase the expression of surface MHC-peptide complexes, RKO cells were treated with 0, 10, 25, 50, 250 and

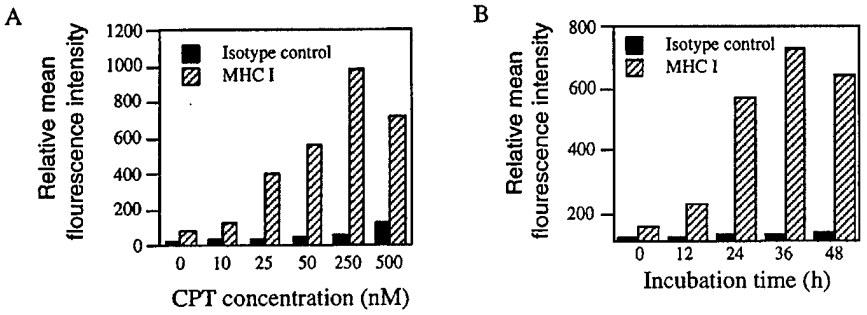

Figure 8 DNA damage increases the expression of surface MHC-peptide complexes on RKO cells when treated with camptothecin in dose- and time-dependent manners. (**a**) RKO cells were treated with 0, 10, 25, 50, 250 and 500 nM camptothecin for 24 h. (**b**) RKO cells were treated with 250 nM camptothecin for 0, 12, 24, 36 and 48 h. The level of surface MHC-peptide complexes was determined with anti-human HLA-ABC antibody B-H9. Mouse IgG1 was used as an isotype control

500 nM camptothecin for 24 h or treated with 250 nM camptothecin for 0, 12, 24, 36 and 48 h. We found that the level of surface MHC-peptide complexes was increased markedly in RKO cells by DNA damage in dose- and time-dependent manners (Figure 8a, b). In contrast, DNA damage had no effect on the MHC class I expression in RE6-26 cells (data not shown). These results suggest that p53 is responsible for the upregulation of the MHC class I pathway following DNA damage.

Discussion

In this study we have demonstrated that TAP1 can be induced by both p53 and several DNA-damaging agents. The induction of TAP1 by DNA damage is p53-dependent because TAP1 is not induced in cells when p53 is functionally null. We found that this induction is mediated by a p53-responsive element located 300 nucleotides downstream of the TAP1 transcription start site. Furthermore, the newly synthesized, p53-induced TAP1 protein is functional in increasing the transport of MHC class I peptides and subsequent expression of surface MHC-peptide complexes.

Since the MHC class I pathway is critical for host tumor surveillance (Pamer and Cresswell, 1998), tumor cells could evade tumor surveillance by acquiring mutations that inhibit the MHC class I pathway. Thus, mutation of one or more of the genes that encode key components for the MHC class I pathway would diminish or abrogate the host tumor surveillance. Indeed, the genes that encode the MHC heavy chain HLA-ABC and light chain β2M were found to be mutated in melanoma tumors (D'Urso *et al.*, 1991; Restifo *et al.*, 1993a). In adenovirus 12-transformed cells, the expression of the LMP2 gene was inhibited by adenoviral oncoproteins (Deiss and Kimchi, 1991; Proffitt and Blair, 1997). Interestingly, mutations that affect TAP1 occur frequently in a variety of human tumors (Amiot *et al.*, 1998; Chen *et al.*, 1996a; Cromme *et al.*, 1994; Kaklamanis *et al.*, 1995; Khanna *et al.*, 1998) and tumor cell lines (Alpan *et al.*, 1996; Johnsen *et al.*, 1998; Restifo *et al.*, 1993a; Vitale *et al.*, 1998; Wang *et al.*, 1998). Here we found that the tumor suppressor p53 is necessary for inducing TAP1 in cells following DNA damage. Thus, a dysfunctional p53 in more than 50% of human tumor cells would not induce TAP1 following genotoxic stress.

How does this novel activity of p53 relate to the central role of p53 in tumor suppression? p53 is a well-defined checkpoint protein in the cell cycle (Almog and Rotter, 1998; Ko and Prives, 1996; Levine, 1997). When cells are exposed to extracellular or intracellular stresses, for example, DNA damage, p53 is stabilized, resulting in cell cycle arrest, apoptosis, or differentiation. Cells suffering from DNA damage often express abnormal cellular proteins that need to be processed and presented on the cell surface (Old and Chen, 1998). These cells are then recognized by the host immune system, leading to their elimination. Our data suggest that p53 also activates the MHC class I pathway by inducing TAP1, which would facilitate this process. If tumor cells acquire additional mutations that inactivate p53, this process of tumor surveillance would be curtailed. Similarly, when oncogenic tumor viruses invade cells, viral proteins are expressed in cells, and then are processed and expressed on the cell surfaces by the MHC class I pathway, leading to elimination of the infected cells (McMichael, 1998; Ploegh, 1998). However, viral oncoproteins, such as HPV E6, adenoviral E1B, and hepatitis B virus (HBV) X, inactivate p53 (Ko and Prives, 1996), which in turn would abrogate the p53-dependent activation of TAP1. We have shown here that HPV E6 oncoprotein does just this in RKO and HCT116 cells. Subsequently, the infected cells would evade recognition by the host immune system and become transformed. Thus, we hypothesize that p53 may have a function in tumor surveillance and inactivation of p53 may be one mechanism that tumor cells use to evade host tumor surveillance.

The MHC class I pathway has been found to be defective in several neuroblastoma cell lines (Cheng *et al.*, 1996), which also carry a hemizygous deletion of a 9 cM interval on chromosome 1p35-36.1 where the p73 gene is located (Kaghad *et al.*, 1997). Since p73 is expressed from only one allele in some cells due to genomic imprinting (Kaghad *et al.*, 1997), a hemizygous deletion of the expressible allele would result in total loss of p73 expression. In this study, we found that p73 is capable of activating the TAP1 gene. Thus,

consistent with the previous observation, loss of p73 may be responsible for down-regulation of the MHC class I pathway in some neuroblastoma cells.

IFN-γ is the most potent inducer of the MHC class I pathway (Stark *et al.*, 1998). Upon binding to its receptor, IFNγ activates the Jak/Stat signaling pathway, leading to induction of at least two groups of transcriptional activators, i.e., the IFN regulatory factors (IRFs) and the class II transactivator (CIITA). IRFs bind to the IFN-stimulated response element (ISRE) and activate several genes in the MHC class I pathway, including the TAP1 gene (Pamer and Cresswell, 1998; Stark *et al.*, 1998). CIITA binds to the site α in the MHC class I heavy chain genes and activates HLA-ABC expression (Gobin *et al.*, 1997; Martin *et al.*, 1997). Since the induction of TAP1 by IFNγ occurs in H1299 cells that are p53-null (Figure 4), the regulation of the MHC class I pathway by IFNγ is independent of p53. A recent report showed that IFNγ-insensitive $p53^{-/-}$ mice develop tumors more rapidly with a broader spectrum of tumors when compared to either $p53^{-/-}$ mice or IFNγ-insensitive mice individually (Kaplan *et al.*, 1998). Furthermore, we found that p53 can cooperate with IFNγ to activate the MHC class I pathway. Thus, it is likely that tumor cells lacking both p53 and an IFNγ response would be defective in the MHC class I antigen presentation pathway, and such cells would become less immunogenic.

Materials and methods

Cell culture

H1299, HCT116, LS174T, LnCap, MCF-7 and WI-38 cell lines were purchased from American Type Culture Collection. RKO cells were cultured as described (Nelson and Kastan, 1994). 8OS14 cell line was cultured as described (Waldman *et al.*, 1996). RE6-26 and HCT116E6 are derivatives of RKO and HCT116, respectively, which were stably transfected with the E6 gene from human papilloma virus (HPV) 16 (Munger *et al.*, 1989). p53-3 and p53(R249S)-2 cell lines, derivatives of H1299 that inducibly express wild-type p53 and p53(R249S), respectively, were cultured as described (Chen *et al.*, 1996b). The H1299 cell lines that inducibly express p73α, p73α292 and p73β are p73α-22, p73α292-20 and p73β-9, respectively, as previously described (Zhu *et al.*, 1998a). The MCF7 cell line, which expresses tet-VP16 for generation of tetracycline inducible cell lines, was purchased from ClonTech (Palo Alto, CA, USA). MCF7 cell lines that express inducible proteins of interest were generated as previously described (Chen *et al.*, 1996b). Camptothecin, doxorubicin, and actinomycin D were purchased from Sigma (St. Louis, MC, USA). Human recombinant IFNγ was purchased from Boehringer Mannheim Biochemical (Germany).

RNA isolation, cDNA subtraction assay, and Northern blot analysis

Poly(A)$^+$ RNA was isolated from p53-3 cells using mRNA purification kit (Pharmacia, Piscataway, NJ, USA). Total RNA was isolated from cells using Trizol reagents (Life Technologies, Inc., Gaithersburg, MD, USA). cDNA subtraction assay was performed using ClonTech PCR-Select cDNA Subtraction kit (ClonTech, Palo Alto, CA, USA). Northern blot analysis was performed as described previously (Zhu *et al.*, 1998a). p21 and GAPDH probes were prepared as described previously (Zhu *et al.*, 1998b). TAP1 probe, a 800-bp *Sma*I–*Hind*III fragment, was prepared from human TAP1 cDNA. LMP2 and TAP2 probes were generated by RT–PCR as described previously (Restifo *et al.*, 1993a). HLA-ABC probe was prepared from mouse H2-K^b cDNA. β_2M cDNA probe (GenBank # AA143790) and LMP7 cDNA probe (AA147042) were purchased from Genome System Inc. (St. Louis, MO, USA).

Electrophoretic mobility shift assay (EMSA) and luciferase assay

Purification of the p53 protein and EMSA were performed as described previously (Chen *et al.*, 1993). The EMSA probe was a 59-bp fragment containing a potential p53-binding site (underlined) in the TAP1 gene: 5′-atcgacgtaagcttctg-cagggcttgg*ccctgccgsggacttgcctagatctacgt-3′. For luciferase assay, the fragment was cloned upstream of a minimal c-*fos* promoter and a firefly luciferase reporter gene (Johansen and Prywes, 1994), and the resulting construct designated TAP1-Fluc. GADD45-Fluc was described previously (Chen *et al.*, 1995). TAP1-Fluc or GADD45-Fluc was co-transfected into H1299 cells with control vector pcDNA3 or a vector that expresses wild-type p53, p53(V143A), p53(R175H), p53(R249S), p53(R273H), p73α or p73β. Dual luciferase assay was performed according to the manufacturer's instructions (Promega).

Western blot analysis

Western blot analysis was performed as described previously (Zhu *et al.*, 1998b). Anti-human TAP1 monoclonal antibody, Ab148.3, was kindly provided by Dr B Seliger (Meyer *et al.*, 1994). Antibodies against p53, p21, actin were described previously (Zhu *et al.*, 1998a).

Peptide, peptide labeling and peptide transport assay

Two MHC class I peptides were synthesized by Molecular Biology Core Facility (Medical College of Georgia) for use in the transport assay. These were: B27, a variant of an HLA-B27-binding, human histone 3 peptide (RRYQ*N*STEL), where Asn is substituted for Lys (Ma *et al.*, 1997); and A3, a variant of an HLA-A3-binding, HIV nef 7B peptide (QVPLR*N*MTYK), where Asn is substituted for Pro (Ma *et al.*, 1997). The peptides were labeled with Na ^{125}I (Amersham Pharmacia) and purified through a sephadex G-25 column. The specific activity of the labeled peptides was approximately 100 c.p.m./fmol. Transport assay was performed as previously described (Ma *et al.*, 1997).

FACS analysis

FACS analysis was performed as previously described (Ma *et al.*, 1997). FITC-labeled mouse anti-human HLA-ABC monoclonal antibody B-H9 was purchased from BioSource International (Carmarillo, CA, USA). FITC-labeled mouse IgG1 monoclonal antibody was purchased from PharMingen (San Diego, CA, USA). The relative amount of the surface MHC-peptide complexes is measured by the relative mean fluorescence intensity from FITC-labeled mouse anti-human HLA-ABC monoclonal antibody.

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The p53 family: same response, different signals?

Xinbin Chen

TP53, the gene that encodes p53, is a well-defined tumor suppressor gene that is frequently mutated in human cancers. Recently, two proteins homologous to p53, termed p73 and p63, were identified. Current data indicate that both p73 and p63, like p53, can induce cell-cycle arrest and apoptosis, suggesting that they might also be tumor suppressors. However, the physiological signals that can regulate p53, for example, DNA damage, have no effect on p73, as tested in several cell lines. Furthermore, the signaling pathways by which p73 (and possibly p63) induces cell-cycle arrest and apoptosis appear to be similar to those of p53, but also have important differences. Thus, the p53 family proteins are closely related but might have distinct physiological functions.

MOST tumor suppressor genes belong to families with several members. For almost two decades, no other *TP53* family member was identified, and it was believed to be an orphan without a family; but no longer. In fact, the *TP53* family has become quite large. Its first relative, *TP73*, which encodes p73, was identified by chance in 1997 (Ref. 1), and its second, *TRP63* (which encodes p63 and is also known as *KET*, *P51*, *P40*, chronic ulcerative stomatitis protein (CUSP) and *P73L*), was identified independently by several groups[2–7]. Furthermore, p53CP, a 40-kDa polypeptide[8], and NBP (non-p53 p53RE binding protein), a 44-kDa polypeptide[9], were also found to be capable of specifically binding to the same DNA element as does p53. Although the genes that encode p53CP and NBP have not yet been cloned, these proteins could be new members of the p53 family or alternatively spliced forms of the existing p53 family proteins.

Human p53 comprises 393 amino acid residues (Fig. 1). The *TP53* gene consists of 11 exons and is located at chromosome 17p13.1. Alternative splicing of intron 9 in human p53 mRNA leads to production of a protein truncated at the C-terminus (p53AS)[10]. It is well documented that mouse p53 and p53AS have different patterns of production and different activities[11,12] but the significance of human p53AS in tumor suppression remains to be elucidated. There are at least four alternatively spliced forms of human p73: p73α, p73β, p73γ and p73δ (Fig. 1)[1,13]. The *TP73* gene contains 14 exons and is located at chromosome 1p36.33 (Ref. 1). The human p63 subgroup of proteins contains the most variants within the p53 family[2–4]. The *TRP63* gene is located at chromosome 3q27–29 and contains 15 exons (Fig. 1)[2,4]. The *TRP63* gene can be transcribed from two different promoters, which are located upstream of exon 1 and within intron 3, respectively[2]. Alternative splicing of p63 mRNA transcribed from the upstream promoter leads to the production of three spliced forms of p63: p63α, p63β and p63γ (Ref. 2). When *TRP63* is transcribed from the **cryptic promoter** in intron 3, three N-terminal truncated proteins, ΔNp63α, ΔNp63β and ΔNp63γ, are produced[2]. A splicing variant that deletes four amino acids in exon 9 was detected in both the p63 and ΔNp63 species[2]. The human KET protein appears to be translated from the alternative start site of the p63α transcript, generating 39 extra residues at the N-terminus[5]. Because the C-terminal 114 amino acids in p40 are different from other p63 variants, p40 is one of the p63 isoforms[3]. Altogether, the *TRP63* gene encodes at least 14 variants of p63.

The p53 protein contains several functional domains (Fig. 2b; Ref. 14 and references therein): **activation domain** 1 (AD1) and AD2, located within residues 1–42 and 43–63, respectively[15,16]; five proline-rich **growth-suppression motifs**[17], located within residues 64–90; a sequence-specific DNA-binding domain, located within residues 100–300; a nuclear localization signal (NLS), located within residues 316–325; an oligomerization domain, located within residues 334–356; and a basic C-terminal regulatory domain, located within residues 363–393. A comparison of p53 sequences from various vertebrates revealed five evolutionarily conserved boxes. Box I is in AD1 (Refs 18,19) and boxes II to V are in the sequence-specific DNA-binding domain. Importantly, the vast majority of p53 missense mutations found in human tumors are clustered in the sequence-specific DNA-binding domain. Indeed, both p73 and p63 were identified as p53 family members because they contain a DNA-binding domain homologous to that of p53 (Refs 1–4).

How do p73 and p63 compare with p53? The homology between p53 and the other family members is extensive within the most conserved p53 functional domains (Fig. 2). Residues 1–59 in p63 and 1–54 in p73 are 22% and 29% identical, respectively, to residues 1–45 of AD1 in p53. Residues 142–321 in p63 and 131–310 in p73 are 60% and 63% identical, respectively, to residues 113–290 of the sequence-specific

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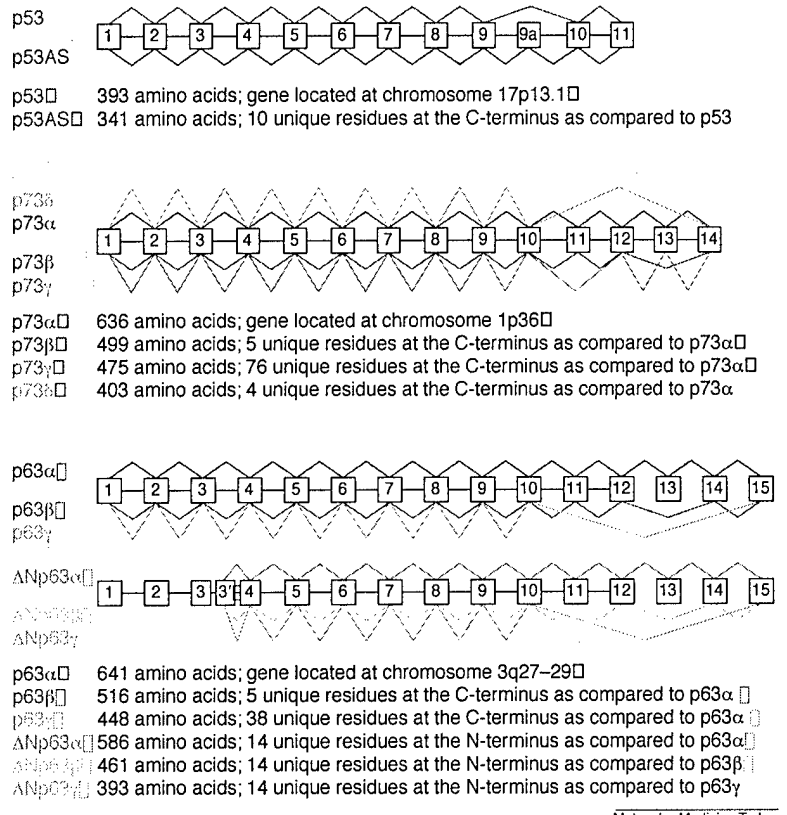

Figure 1. Gene organization of the P53 family members. Exons are shown as numbered boxes and introns as lines. TRP63 is also known as KET, P51, P40, CUSP and P73L. The p51B protein is identical to p63α, p51A identical to p63γ, p73L and CUSP identical to ΔNp63α, p40 identical to ΔNp63 except the C-terminal 114 amino acids, and KET identical to p63α except the N-terminal 39 amino acids. Modified from Refs 1,2,13.

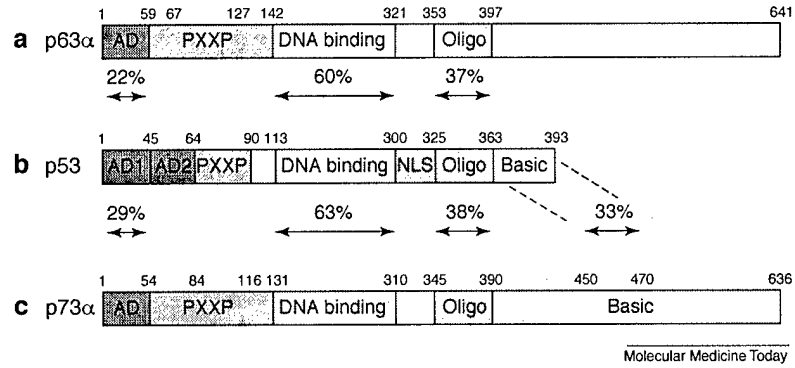

Figure 2. Homology among the p53 family of proteins, (**a**) p63α, (**b**) p53 and (**c**) p73α. Numbers above double-arrowed bars represent the percentage of p53-identical residues found in p63 or p73. Numbers above the bars are amino acid numbers. Abbreviations: AD, **activation domain**; Basic, p53 C-terminal basic domain; DNA binding, sequence-specific DNA-binding domain; NLS, nuclear localization signal; Oligo, oligomerization domain; **PXXP**, proline-rich domain where P represents proline and X any amino acid.

DNA-binding domain in p53. Residues 353–397 in p63 and 345–390 in p73 are 37% and 38% identical, respectively, to residues 319–363 of the oligomerization domain in p53. Although homology in the **PXXP motifs** between p53 and other p53 family members is not significant, both p63 and p73 do contain two **PXXP motifs**. Interestingly, residues 450–470 in p73 are 33% identical to the C-terminal basic regulatory domain in p53, while such a domain is not present in p63. It should be mentioned that p63 and p73 are more homologous to each other than to p53. Overall, p63 is 53% identical to p73. Specifically, the identity between p63 and p73 is 30% in the **activation domain**, 87% in the DNA-binding domain and 65% in the oligomerization domain[2-4]. Furthermore, human and murine p63 are 99% identical, with only eight substitutions, whereas human and mouse p53 are only 77% identical. Phylogenetic analysis of p53, p63 and p73 indicated that p63 is the most primitive and ancient member of the p53 family, suggesting that p63 might in fact be the evolutionary ancestor of the p53 family[2].

Are *TP73* and *TRP63* tumor suppressor genes?

TP53 is a bona fide tumor suppressor gene because it fulfills the 'classical features' of tumor suppressors (Table 1)[20], namely: (1) loss of function mutations accompanied by loss of heterozygosity occur in tumors; (2) in Li–Fraumeni syndrome, which predisposes individuals to multiple early-onset cancers, one allele of *TP53* is constitutively mutated; (3) *TP53* mutations occur in ~50% of spontaneous human tumors; (4) overexpression of *TP53* inhibits the growth of transformed cells; and (5) p53-deficient mice develop tumors at an early age.

TP73 was initially classified as a possible tumor suppressor gene because it is related to *TP53*, it maps to chromosome 1p36.33, a region frequently deleted in neuroblastoma and other human cancers and it has been found to be monoallelically expressed owing to **genomic imprinting** (Table 1)[1]. Thus, hemizygous deletion of the 'expressable' allele would result in total loss of *TP73* expression in cells. However, the status of *TP73* as a tumor suppressor gene has been challenged by recent observations[21-26]. Notably, *TP73* can be biallelically expressed in both normal and tumor tissues or cell lines, including neuroblastoma; and mutation of the *TP73* gene occurs infrequently in human cancers[27,28]. However, not all tumor suppressor genes fulfill the classical features mentioned above. Among these is *CDKN2D*, which encodes $p19^{ARF}$ and is an

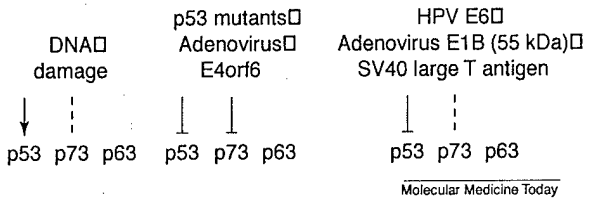

Figure 3. Modulation of the p53 family members by DNA damage, p53 mutants and viral oncoproteins. Arrow indicates activation. Blocked line indicates inhibition. Dashed line indicates no effect. Absence of line indicates not examined. It should be mentioned that although p73 is not induced by DNA damage and ultraviolet radiation in several neuroblastoma and non-neuroblastoma cell lines[1], it is still possible that some forms of DNA damage might affect p73 activity in certain tissues. In addition, more studies are needed to clarify the contradictory effect of the adenoviral E4orf6 oncoprotein on p73 (Refs 41–43) and to determine whether TP53 mutants other than TP53(R175H) and TP53(R248H) can inhibit p73 activity. Abbreviation: **HPV, human papillomavirus.**

alternatively spliced form of the *CDKN2A* tumor suppressor gene, which encodes $p16^{INK4a}$ (Ref. 29). Although homozygous deletions of *CDKN2D* occur in a wide range of human tumors, inactivating point mutations have not been found in the unique exon 1β of *CDKN2D*, which encodes the N-terminal 64 amino acids necessary for and sufficient to induce cell-cycle arrest[30,31]. Moreover, many inactivating point mutations found in *CDKN2A* are also predicted to alter *CDKN2D*, but those that have been tested experimentally do not affect the ability of $p19^{ARF}$ to induce cell-cycle arrest[30,31]. Interestingly, the mechanism by which *CDKN2D* functions as a tumor suppressor gene is its ability to regulate p53 function. Because *TP53* is frequently mutated, there might be no selective pressure for a mutated *CDKN2D*. In addition, p73 function can be inhibited by two tumor-derived *TP53* mutants, *TP53*(R175H) and *TP53*(R248H), in mammalian cells (Fig. 3)[32]. Therefore, in a similar manner, tumor cells with a mutated *TP53* gene would also have no selective pressure to mutate the *TP73* gene. Nevertheless, more studies are needed to determine whether *TP73* is a true tumor suppressor gene.

It is also not certain whether *TRP63* is a tumor suppressor gene (Table 1). *TRP63* is located at chromosome 3q27–29, a region that is not a common site of loss of heterozygosity in human cancers. The *TRP63* gene was found to be mutated, albeit infrequently, in both human tumor tissues and cancer cell lines[4]. Further complicating this matter is the observation that although p63 might have functions similar to those of p53 in cell-cycle arrest and apoptosis, ΔNp63, which lacks an **activation domain**, inhibits the activity of both p53 and p63, thereby exhibiting oncogenic functions[2]. Interestingly, *TRP63* is highly expressed in the basal region of many epithelial tissues[2] and is essential for limb, craniofacial and epidermal morphogenesis[33,34]. Therefore, to determine whether p63 plays any role in tumorigenesis will require extensive genetic and biochemical analyses.

Modulation of the p53 family proteins

Genomic instability is central to the development of cancer, and p53, by regulating the normal cellular response to DNA damage and other cellular insults, plays an essential role in the control of growth and division, thereby serving as a 'guardian of the genome'[35]. It is well documented that upon DNA damage, or under conditions of hypoxia or other cellular stresses, the p53 protein is stabilized (Fig. 3) and accumulation of the p53 protein leads to activation of checkpoint-control responses (for comprehensive reviews on this topic, see Refs 14,36–38). Although it is still not certain how p53 is stabilized, one mechanism for such a process is that p53 can be phosphorylated by a DNA-damage-inducible kinase, **ataxia telangiectasia-mutated (ATM)** kinase[39], and the phosphorylated p53 is then resistant to ubiquitin-dependent proteolysis. In contrast, p73 is not induced in several cell lines when treated with DNA-damaging agents, actinomycin D and doxorubicin, as well as ultraviolet and ionizing radiation[1]. The response of p63 to DNA damage remains to be determined.

p53 was originally identified as a protein that binds to the SV40 virus large T antigen[14,37]. It is believed that the physical interaction with and inactivation of p53 by viral oncoproteins, such as the SV40 large T antigen, adenovirus E1B 55-kDa protein and **human papillomavirus (HPV)** E6 protein, plays a central role in viral tumorigenesis[14,37]. However, recent experiments failed to show a physical interaction of p73 with the adenovirus E1B 55-kDa protein, **HPV** E6 protein and SV40 large T antigen both *in vitro* and *in vivo* (Fig. 3)[40–43]. Thus, these viral oncoproteins do not inhibit p73 function and the stable binding of these viral oncoproteins to p73 is apparently not necessary for transformation. The E1B 55-kDa protein can associate with p53, but not with p73, owing to the presence of an E1B 55-kDa-binding domain in p53, which is not present in p73 (Ref. 42). In domain-swapping experiments, five residues present in p53 (24-KLLPE-28), but not in the equivalent positions in p73 (20-SSLEP-24), were found to be necessary for E1B 55-kDa protein binding. However, one viral oncoprotein, adenovirus

Table 1. Members of the p53 family: how do they weigh up as tumor suppressors?

Characteristic[a]	p53	p63	p73
Loss-of-function mutations and LOH	Yes	Not found	Imprinted?[b]
Mutations in inherited cancer syndromes	Li–Fraumeni[18]	Not found	Not found
Somatic mutations in spontaneous tumors	Yes	Rare[c]	Rare[d]
Growth inhibition (cell-cycle arrest and apoptosis)	Yes	Yes[e]	Yes
Phenotype of mutant mice	Developmentally normal[64]; susceptible to spontaneous tumors	Postnatal lethal[33,34]; limb, craniofacial and epidermal morphogenesis	Not done

[a]The characteristics that define a tumor suppressor gene are taken from Ref. 20.

[b]p73 was found to be expressed monoallelically[1] and biallelically[21–25]. Loss of heterozygosity (LOH) for p73 occurs frequently in neuroblastomas[1,27].

[c]One somatic p63 missense mutation found in 66 human primary tumors[4].

[d]Mutations in p73 occur but infrequently[27,28].

[e]p63 but not ΔNp63 can induce cell-cycle arrest and apoptosis[2].

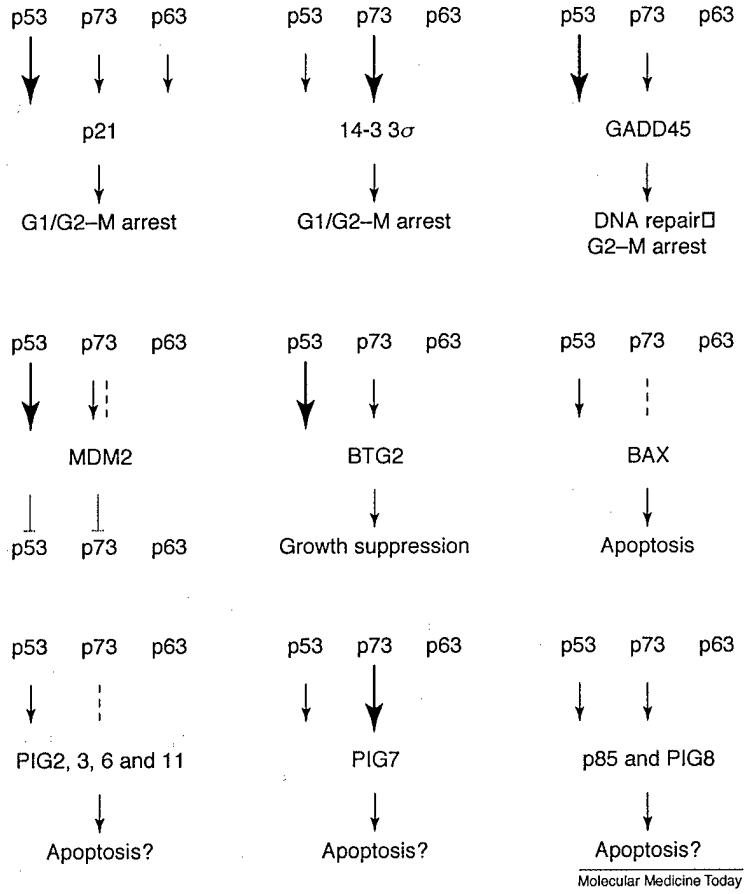

Figure 4. Regulation of the cellular target genes by the p53 family proteins. Heavy arrow indicates strong activation. Light arrow indicates weak activation. Blocked line indicates inhibition. Dashed line indicates no effect. Absence of line indicates not examined. Data obtained from Refs 1,2,4,44,45,62,63. Abbreviations: BTG2, B-cell translocation gene 2 antiproliferative; MDM2, human homolog of the murine double minute 2 gene; PIG, p53-induced gene.

Glossary

Activation domain – A region of a transcription factor that is required for its function. It might directly or indirectly interact with the basal transcription machinery, facilitating its assembly.

Ataxia telangiectasia-mutated (ATM) gene – A gene mutated in the autosomal recessive disorder ataxia telangiectasia (AT). The gene product is a member of the phosphoinositide 3-kinase (PI 3-kinase) family.

Cryptic promoter – Also called an alternate promoter; a DNA sequence that can control RNA transcription in certain tissues or in response to certain physiological stimuli.

Genomic imprinting – A phenomenon whereby a gene on the paternally and maternally derived chromosomes is differentially expressed.

Growth-suppression motif – A protein domain that is necessary for inhibiting cell proliferation; for example, the PXXP motif in p53.

Human papillomavirus (HPV) E6 – An HPV-encoded oncoprotein that can bind to p53 and facilitate ubiquitin ligation to p53, leading to degradation of p53.

PXXP motif – A motif that can bind to SH3 (Src homology 3) domains; P represents proline and X any amino acid.

E4orf6, was capable of associating with p73 and inhibiting the activity of p73 in one experimental protocol[41,43], but not in another[42]. Thus, further studies are needed to address this issue. Whether these viral oncoproteins can regulate p63 remains to be determined.

The signaling pathways of the p53 family proteins leading to tumor suppression

Like p53, p73 and p63 can induce cell-cycle arrest and apoptosis[1,2,4,44,45], both of which constitute the basic cellular mechanisms by which p53 mediates tumor suppression; and like p53, p73 and p63 are transcription factors[1,2,4,44]. Loss of p73 transcriptional activity abrogates its activity in cell-cycle arrest and apoptosis[1,44,45]. It is well known that p53 upregulates p21, an inhibitor of the cyclin-dependent kinases[46–48]. This is primarily responsible for p53-dependent arrest in G1 phase. Consistent with their ability to induce cell-cycle arrest, both p73 and p63 can induce p21 synthesis (Fig. 4)[1,4,44,45]. Although p73 can induce p21, the level of cellular p21 induced by p73 is several times lower than that induced by p53 (Ref. 45). Two other p53 target genes that can cause growth suppression and might be involved in cell-cycle arrest, *GADD45* (Ref. 49) and B-cell translocation gene 2 antiproliferative (*BTG2*; Ref. 50), are only weakly activated by p73 (Fig. 4)[45]. Therefore, it remains to be determined whether the level of p21 induced by p73 is sufficient to cause cell-cycle arrest and whether other cellular genes might also be involved in p73-dependent cell-cycle arrest.

p53-dependent G2–M arrest is mediated, at least in part, by upregulation of a gene known as *14-3-3σ* (Ref. 51). The product of this gene interacts with the cdc25 phosphatase to block activation of the cyclin B-dependent cdc2 kinase, which is required for initiation of mitosis[52]. Consistent with the observation that p73 can induce G2–M arrest, p73 is capable of inducing *14-3-3σ* (Fig. 4)[45]. Interestingly, p73 induces several-fold higher levels of the *14-3-3σ* gene product than does p53. These results suggest that a signaling pathway to induce arrest in G2–M is conserved between p53 and p73, and that *14-3-3σ* might be a *bona fide* cellular target gene of p73, even though it was originally identified as a potential p53 target gene. It remains to be determined whether p63 can induce *14-3-3σσ* and cell-cycle arrest in G2–M.

The outstanding questions

- What are the physiological signals that regulate p63 and/or p73? Do the signals that regulate p53, such as DNA damage, hypoxia and nucleotide deprivation, also regulate p63 and p73?
- Are there cellular target genes that are regulated specifically by p63 or p73? What are the common targets among the p53 family members?
- What are the domains in p63 and p73 necessary for growth suppression? Are the domains in p63 and p73 separable for cell-cycle arrest and apoptosis?
- Do functional interactions exist among p53, p63 and p73 in cells under physiological conditions?
- Is there a possibility that activating p63 and p73 might be a useful therapeutic strategy for tumors that have lost p53 activity?

Although both p53 and p73 can induce apoptosis[44,45], the signaling pathways used might differ, based on the differential ability of p73 to activate some p53 target genes. *BAX* and several redox-related genes [p53-induced gene 2 (*PIG2*), *PIG3*, *PIG6* and *PIG11*] that might be involved in mediating p53-dependent apoptosis[53,54] were not significantly induced by p73 (Fig. 4)[45]. Although *PIG7*, *PIG8* and *P85* were induced by P73 (Fig. 4)[45], the functions of *PIG7* and *PIG8* in apoptosis are still unknown and the role of *P85* in apoptosis appears to be restricted to the cellular response to oxidative stress[55]. Because p73 transcriptional activity is required for inducing apoptosis[1,44,45], it is possible that a distinctive group of cellular genes that can be activated by p73 might be responsible for mediating apoptosis. The signaling pathway for p63 induction of apoptosis remains to be determined.

The human homolog of the murine double minute 2 gene (*MDM2*), an oncogene that negatively regulates p53 and is also induced by p53 (Ref. 56), is weakly induced by p73β but not by p73α (Fig. 4)[45]. MDM2 binds to p53, enhancing the degradation of p53 through the ubiquitination pathway[57–60], as well as concealing the **activation domain** of p53 (Ref. 61), thus abolishing its ability to regulate transcription. Interestingly, MDM2 binds to and suppresses p73 function without promoting p73 degradation[62,63]. The domain required for MDM2 binding is present in the **activation domain** of p63 (Ref. 2), but whether p63 can regulate *MDM2* or be regulated by MDM2 remains to be elucidated.

Prospects for the future

Among the most pressing issues is the identification of physiological signals that regulate p63 and/or p73 activity. In addition, dissecting the domain(s) necessary for p63- and p73-dependent cell-cycle arrest and apoptosis might also provide insights into how p63 and p73 are regulated by physiological conditions and might be informative for engineering a more potent p63 or p73 for use as a therapeutic agent. Because p63 and p73 are infrequently mutated in human cancers, activating p63 and/or p73 pathways in cells that have lost p53 activity might be a useful therapeutic strategy. Furthermore, it will be interesting to determine whether and how p63 or p73 cooperate with p53 to mediate tumor suppression; this will guide future decisions as to whether p63 or p73 should potentially be used with p53 for gene therapy.

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Late-breaking news

Since acceptance of this manuscript, the signals that can regulate and modify p73 have been unraveled. p73 can be stabilized by DNA damage in a c-Abl-dependent manner when cells are treated with cisplatin[1] and phosphorylated at a tyrosine residue by c-Abl when cells are gamma-irradiated[2,3]. However, it is not clear why p73 is neither phosphorylated when cells are treated with cisplatin nor stabilized when cells are irradiated with ultraviolet light[1]. Nevertheless, c-Abl directly transduces the DNA damage signals to p73 through its Src homology 3 domain, which interacts with the C-terminal PXXP motif of p73 (Refs 2,3). Both stabilization and tyrosine phosphorylation of p73 by c-Abl enhance the transcriptional and pro-apoptotic activity of p73 (Refs 1–3). Therefore, future studies should address whether other physiological signals that induce p53, such as hypoxia and nucleotide deprivation, can also induce p73.

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Appendix 10

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Aquaporin 3, a glycerol and water transporter, is regulated by p73 of the p53 family

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Abstract p73, a member of the p53 family, has been shown to exhibit similar biochemical activities to that of p53. However, in contrast to p53, p73 is rarely mutated in human tumors and p73 mutant mice develop neurological, pheromonal, and inflammatory defects, but not spontaneous tumors. Furthermore, p73 mutant mice are deficient in the physiological control of cerebral spinal fluid. To determine what mediates these p73 activities, cDNA subtraction assay was performed to identify cellular genes that are regulated by p73. We found that aquaporin 3 (AQP3), a glycerol and water transporter, is regulated by p73. In addition, we identified a potential p53 response element in the promoter of the AQP3 gene, which is responsive to p73. This suggests that AQP3 may mediate the activity of p73 in maintaining cerebral spinal fluid dynamics. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: p73; p53; AQP3; Glycerol; Water transporter

1. Introduction

p73, a member of the p53 family, shares a considerable sequence similarity with p53, especially in the transactivation, DNA binding, and tetramerization domains [1]. p73 is expressed as at least six different splicing variants: p73α, p73β, p73γ, p73δ, p73ε, and p73ζ [2,3]. A ΔN variant of these p73 proteins has been found, which lacks the N-terminal activation domain and is potentially dominant negative [4]. Like p53, p73 can bind to the p53 response element and regulate some p53 target genes [2,3]. p73 can also induce cell cycle arrest and apoptosis [2,3]. However, unlike p53, p73 is not frequently mutated and p73 mutant mice are not susceptible to spontaneous tumors [2–4]. Instead, p73 is necessary for the normal neurological and pheromonal development and the inflammatory response [4]. Mutant p73 mice are also abnormal in maintaining spinal fluid dynamics [4].

The aquaporins are a family of small transmembrane water and/or glycerol transporters. Currently, ten aquaporins (AQP0–AQP9) have been identified and are divided into two groups, 'aquaporins' and 'aquaglyceroporins'. The aquaporin group, including AQP0, 1, 2, 4, 5, 6, and 8, is structurally related to the bacterial water channel and highly selective for the passage of water [5–8]. The aquaglyceroporin group, including AQP3, 7, and 9, is structurally related to the bacterial glycerol facilitator with an enlarged loop E and permeated by water, glycerol, and some other solutes [5–8]. AQP3 is relative weak in the transport of water, but very efficient in the transport of glycerol and other solutes [9–12]. AQP3 is initially cloned from the kidney collecting duct and brain meningeal cells [9,10,13] and later found to be expressed in red and dendritic cells and epithelium cells from a variety of tissues, including tracheal, eye, nasopharyngeal, digestive tract, and skin [14–18]. Like AQP2 mutant mice, mice deficient in AQP3 are susceptible to nephrogenic diabetes insipidus but developmentally normal [19]. In an effort to identify cellular genes that mediate p73 activity, we performed cDNA subtraction assay and found that AQP3 is regulated by p73.

2. Materials and methods

2.1. Cell culture and cell lines

MCF7 and H1299 cell lines were purchased from American Type Culture Collection (Rockville, MD, USA). p53-24, p53(R249S)-25, p73α-2, p73α292-3 and p73β-38, derivatives of the MCF7 cell line that inducibly express wild-type p53, p53(R249S), p73α, p73α292, and p73β, respectively, were generated as previously reported [20].

2.2. RNA isolation, cDNA subtraction assay, and Northern blot analysis

Poly(A)+ RNA was isolated from p73β-38 cells using an mRNA purification kit (Pharmacia, Piscataway, NJ, USA). Total RNA was isolated from cells using Trizol reagents (Life Technologies, Inc., Gaithersburg, MD, USA). The cDNA subtraction assay was performed using the Clontech PCR-select cDNA subtraction kit according to the manufacturer's instructions (Clontech, Palo Alto, CA, USA). Subtracted cDNA fragments were cloned into pGEM-T vector (Promega, Madison, WI, USA). Northern blot analysis was performed as described previously [20]. p21 and GAPDH probes were prepared as described previously [20]. A 615-bp *Not*I fragment, prepared from AQP3 cDNA [21], was used to detect AQP3.

2.3. Luciferase assay

A 36-bp fragment (5′-AAGCTAGGTCACCAGCCATGTTCAACAGGCATGTGC-3′) that contains the potential p53 response element in the AQP3 promoter was synthesized and cloned upstream of a minimal c-*fos* promoter and a firefly luciferase reporter gene [22]. The resulting construct was designated AQP3-Fluc. A 990-bp fragment that contains the potential p53 response element and a 790-bp fragment that lacks the potential p53 response element in the AQP3 promoter were generated by PCR and cloned into a promoterless luciferase reporter vector (pGL2-basic) (Promega, Madison, WI, USA). The resulting constructs were designated APP990 and APP790. 2 μg of AQP3-Fluc, APP990, or APP790 was cotransfected into H1299 cells with 1 μg pcDNA3 control vector or a vector that expresses p53, p53(R249S), p73α, p73α292, p73β, or p73β292. 0.1 μg of renilla luciferase assay vector, pRL-CMV (Promega), was also cotransfected as an internal control. The dual luciferase assay was performed according to the manufacturer's instructions (Promega).

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2.4. Immunocytochemistry

Cells were plated on four-chamber tissue culture slides. After washing with PBS, the cells were fixed with 10% formalin, permeabilized with 1% NP-40, and incubated in 15% goat or rat serum to block nonspecific binding. The cells were then stained with anti-p73 (Ab-2, Oncogene, Cambridge, MA, USA) or anti-AQP3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody and examined by fluorescence microscope.

3. Results

To identify novel target genes regulated by p73, the Clontech PCR-select cDNA subtraction assay was performed using mRNA isolated from the p73β-38 cell line, a derivative of the MCF7 cell line that inducibly expresses p73β under the control of a tetracycline-regulated promoter. Several cDNA fragments that may represent genes induced by p73 were isolated. After DNA sequencing, one subtracted cDNA fragment was found to be derived from the aquaporin (AQP) 3 gene. To confirm that AQP3 can be induced by p73, Northern blot analysis was performed using AQP3 cDNA as probe. We found that AQP3 was strongly induced in p73β-38 cells when p73β was expressed (Fig. 1, AQP3 panel, compare lanes 5 and 6). AQP3 was also induced by p73α (Fig. 1, AQP3 panel, compare lanes 1 and 2) and weakly induced by p53 (Fig. 1, AQP3 panel, compare lanes 7 and 8). In contrast, mutant p73α292 and p53(R249S) were incapable of inducing AQP3 (Fig. 1, AQP3 panel, compare lanes 3, 4 and 9, 10). As a control, we tested the expression of p21, a well-defined target gene for both p53 and p73. We found that p21 was induced by p53, p73α, p73β (Fig. 1, p21 panel), but not by mutant p53(R249S) and p73α292 (Fig. 1, p21 panel).

To determine whether AQP3 is transcriptionally regulated by p73, we searched for a potential p53 response element that can be regulated by p73. To do this, we screened a human bacterial artificial chromosome (BAC) library (The Genome System, St. Louis, MO, USA) and identified a clone that contains the entire AQP3 locus. A region of 3570 nucleotides in the promoter of the AQP3 gene was sequenced (data not shown). We found one potential p53 response element (Fig. 2A). This sequence (AAG CTAG *g*TC acc AG*c* CATG TTC aac AGG CATG T*g*C) contains three half sites with only one mismatch each (in bold italics) in the non-critical region as compared to the consensus p53 response element [23,24].

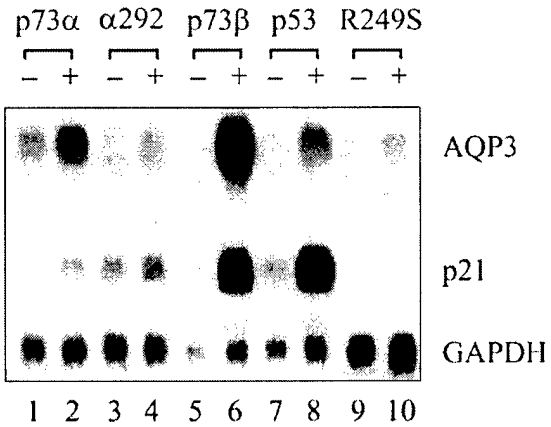

Fig. 1. Upregulation of AQP3 by p73. Northern blots were prepared using 10 μg of total RNA isolated from p73α-2, p73α292-3, p73β-38, p53-24, or p53(R249S)-25 cells under both the uninduced (−) and induced (+) conditions. The blots were probed with cDNAs derived from the AQP3, p21, and GAPDH genes, respectively.

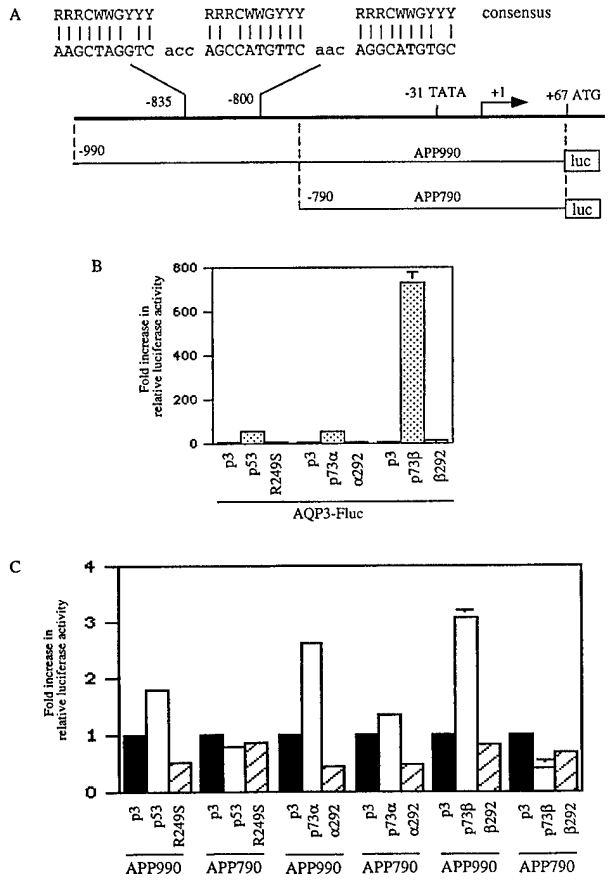

Fig. 2. Identification of a potential p53 response element in the AQP3 gene. A: Schematic representation of the AQP3 genomic structure. The position of the AQP3 transcription and translation start sites and a potential p53 response element are indicated. Shown above the genomic structure are the sequence of the potential p53 response element and the previously characterized consensus response element. R represents purine, Y pyrimidine, and W adenine or thymidine. Shown below the genomic structure is the location of the DNA fragments used to generate two reporter constructs. B: The potential p53 binding site in the AQP3 gene is responsive to p53 and p73, but not to mutant p53 and p73. 2 μg of AQP3-Fluc was cotransfected into H1299 cells with 1 μg of pcDNA3 control vector or a vector that expresses p53, p53(R249S), p73α, p73α292, p73β, or p73β292. The fold increase in relative luciferase activity is a product of the luciferase activity induced by p53 or p73 divided by that induced by pcDNA3. C: The AQP3 promoter that contains the potential p53 binding site is responsive to p53 and p73, but not to mutant p53 and p73. The experiment was performed as in B.

To analyze whether this element is responsive to p53 and p73, we cloned a 36-bp fragment containing the potential p53 response element upstream of the c-*fos* minimal promoter and a luciferase reporter gene [22] to generate a reporter vector, designated AQP3-Fluc. AQP3-Fluc was cotransfected into H1299 cells with either a pcDNA3 control vector or a vector expressing p53, p53(R249S), p73α, p73α292, p73β, or p73β292. We found that the luciferase activity for AQP3-Fluc was markedly increased by p53 (52-fold), p73α (52-fold), and p73β (731-fold), but not by mutant p53(R249S), p73α292, and p73β292 (Fig. 2B).

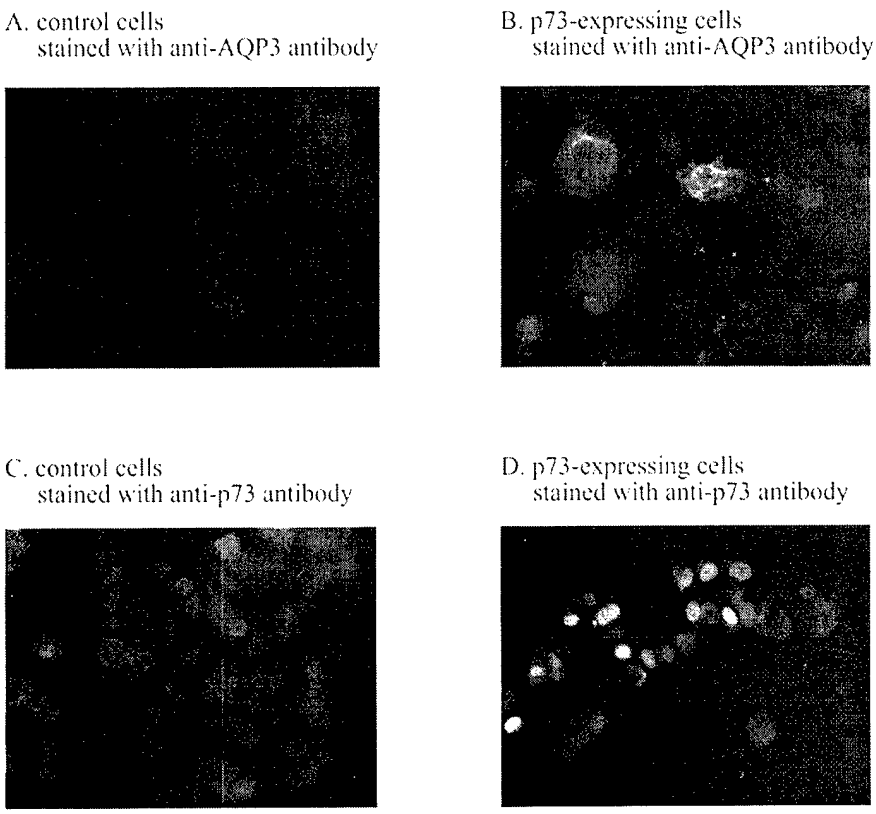

Fig. 3. Immunological detection of the AQP3 protein in p73-expressing cells. p73β-38 cells were uninduced or induced for 24 h, fixed in 4% formalin, and stained with anti-AQP3 or anti-p73 antibody. A: Control cells stained with anti-AQP3 antibody. B: p73β-expressing cells stained with anti-AQP3 antibody. C: Control cells stained with anti-p73 antibody. D: p73β-expressing cells stained with anti-p73 antibody.

To determine whether p53 and p73 can regulate the potential p53 response element in the natural promoter of the AQP3 gene, we cloned the promoter region of the AQP3 gene into a promoterless luciferase reporter vector (pGL2-basic). The resulting vectors are designated APP990 and APP790 (Fig. 2A). APP990, but not APP790, contains the potential p53 response element. We found that the luciferase activity for APP990, but not for APP790, was increased by p73α (2.6-fold), p73β (3.1-fold), and p53 (1.7-fold) (Fig. 2C). In contrast, mutant p53(R249S), p73α292, and p73β292 were incapable of increasing the luciferase activity for both APP990 and APP790. It should be mentioned that the ability of p73β to increase the luciferase activity for both AQP3-Fluc and APP990 was much higher than that by p53 and p73α, consistent with the strong induction of endogenous AQP3 by p73β (Fig. 1).

To determine whether the enhanced expression of the AQP3 gene leads to an increased expression of the AQP3 protein in p73β-expressing cells, we measured the level of the AQP3 protein in cells by immunofluorescence microscopy. We found that the level of the AQP3 protein was substantially increased in p73β-expressing cells as compared to control cells (Fig. 3, compare A and B). We also found that the AQP3 protein was localized on the inner plasma membrane (Fig. 3B), as previously reported [17,25,26]. As a control, we found that p73β was expressed in the nucleus only when induced (Fig. 3, compare C and D).

4. Discussion

Several studies have shown that AQP3 can be regulated by the corticosteroid dexamethasone, activated protein kinase C, and cystic fibrosis transmembrane conductance regulator in airway epithelial cells and lung carcinoma A549 cells [21,26,27]. However, the mechanism by which AQP3 is regulated is still not clear. In this study, we found that AQP3 is strongly induced by p73 and weakly induced by p53. In addition, we have identified a potential p53 response element in the promoter of the AQP3 gene, which is responsive to p73 and p53.

Although p73 and p53 are highly similar in the DNA binding domain, we and others have shown that p73 and p53 differentially regulate p53 target genes [28–30]. For example, 14-3-3σ is strongly induced by p73 whereas p21 and MDM2 are strongly induced by p53. Thus, AQP3 becomes another example. Since the cellular target genes are responsible for the activities of the p53 family members, the differential regulation of cellular genes by the p53 family members may correlate with the distinct activities of p53 and p73 [2,3]. Therefore, AQP3, as a p73 target, may mediate the activity of p73 in regulating the homeostasis of cerebral spinal fluid. In mice, p73 is highly expressed in the epithelial cells of the choroid plexus and the ependymal cells lining the ventricles [4], both of which participate in regulating cerebral spinal fluid dynamics. Therefore, lack of p73 leads to defects in production or reabsorption of cerebral spinal fluid, resulting in hydrocepha-

lus [4]. Among the aquaporin family, AQP3 and AQP4 are expressed in the brain ependymal cells [10,14]. Since AQP4 is not induced by p73 (data not shown), further studies are needed to determine whether lack of AQP3 induction by p73 contributes to the hydrocephalus in the p73 mutant mice.

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Advances in Brief

The Potential Tumor Suppressor p73 Differentially Regulates Cellular *p53* Target Genes[1]

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Abstract

p73, a potential tumor suppressor, is a p53 homologue. Transient over expression of p73 in cells can induce apoptosis and *p21*, a cellular p53 target gene primarily responsible for p53-dependent cell cycle arrest. To further characterize the role of p73 in tumor suppression, we established several groups of cell lines that inducibly express p73 under a tetracycline-regulated promoter. By using these cell lines, we found that p73 can induce both cell cycle arrest and apoptosis. We also found that p73 can activate some but not all of the previously identified p53 cellular target genes. Furthermore, we found that the transcriptional activities of p53, p73α, and p73β to induce their common cellular target genes differ among one another. These results suggest that p73 is both similar to and different from p53 in their signaling pathways leading to tumor suppression.

Introduction

Cytogenetic and molecular genetic analyses have shown that loss of heterozygosity in chromosome 1p36 was frequently found in neuroblastoma (1). Recently, p73, a homologue of the well-defined tumor suppressor p53 (2), was found to lie within the chromosome 1p36 region, suggesting that it may be a new tumor suppressor (2). The *p73* gene is expressed as p73α, a 636-amino acid polypeptide, and p73β, a 499-amino acid poplypeptide that is encoded by an alternatively spliced transcript lacking the 96 nucleotides corresponding to exon 13. The homology between p53 and p73 is extensive within the most conserved p53 domains (*i.e.*, activation, sequence-specific DNA binding, and oligomerization).

As a p53 homologue, it is reasonable to suggest that p73 may have biological activities similar to that of p53. Indeed, p73 was shown to be capable of activating the *p21* cyclin-dependent kinase inhibitor gene (2, 3), a well-defined cellular p53 target primarily responsible for p53-dependent G_1 arrest (4). However, it is not clear whether p73 can induce other cellular p53 targets that also cause growth suppression or cell cycle arrest, *e.g.*, 14-3-3σ (5) and BTG2 (6). Apoptotic activity is the other function that is conserved between p53 and p73, although it is not clear how p73 induces apoptosis. Although several hypotheses for the mechanism of p53-dependent apoptosis have been proposed, it is still not certain how p53 induces apoptosis (7). Many studies show that p53 transcriptional activity contributes to its ability to induce apoptosis (7). There are several candidate genes that play roles in apoptosis that can be activated by p53 *e.g.*, *BAX* (8), *KILLER/DR5* (9), several redox-related genes (*PIG*s; Ref. 10), and the p85 regulatory subunit of the signaling protein phosphatidyl-3-OH kinase (11). In this study, we provide evidence that despite the high similarity in the activation and DNA binding domains between p53 and p73, the p53 and p73 signaling pathways leading to tumor suppression are both similar and different.

Materials and Methods

Plasmids. cDNAs for p73α, p73β, and p73α292 (Ref. 3; kindly provided by W. Kaelin) were cloned separately into a tetracycline-regulated expression vector, 10-3, at its Eco RI and Xba I sites, and the resulting plasmids were used to generate cell lines that inducibly express p73. p73 proteins were tagged at their N-termini with influenza hemagglutinin peptide.

Generation of H1299 Cell Lines that Inducibly Express p73. H1299 cell lines that inducibly express p73 were generated as described previously (12). Individual clones were screened for inducible expression of p73 protein by Western blot analysis using monoclonal antibody 12CA5. The H1299 cell line that inducibly expresses wild-type p53 is p53-3 as described previously (12).

Western Blot Analysis. Cells were collected from plates in PBS, resuspended with 1 × sample buffer, and boiled for 5 min. Western blot analysis was performed as described previously (12). Affinity purified anti-actin polyclonal antibodies was purchased from Sigma Chemical Co. (St. Louis, MO). 12CA5 was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Growth Rate Analysis. To determine the rate of cell growth, cells were seeded at approximately 7.5×10^4 cells/60-mm plate, with or without tetracycline (1 μg/ml). The medium was replaced every 72 h. At times indicated, three plates were rinsed with PBS twice to remove dead cells and debris. Live cells on the plates were trypsinized and collected separately. Cells from each plate were counted three times using the Coulter cell counter. The average number of cells from three plates were used for growth rate determination.

FACS Analysis. Cells were seeded at 2.0×10^5/90-mm plate, with or without tetracycline. Three days after plating, both floating dead cells in the medium and live cells on the plate were collected and fixed with 2 ml of 70% ethanol for at least 30 min. The fixed cells were centrifuged and resuspended in 1 ml of PBS solution containing 50 μg/ml each of RNase A (Sigma Chemical Co.) and propidium iodide (Sigma Chemical Co.). The stained cells were analyzed in a FACS[4] sorter (FACSCaliber; Becton Dickinson) within 4 h. The percentage of apoptotic cells containing a sub-G_1 DNA content was quantitated using the CellQuest program. The percentage of live cells in G_0-G_1, S, and G_2-M phases was quantitated using the ModFit program.

RNA Isolation and Northern Blot Analysis. Total RNA was isolated using Trizol reagents (Life Technologies, Inc.). Northern blot analysis was performed as described (13). The p21, MDM2, BAX, GADD45, and glyceraldehyde-3-phosphate dehydrogenase probes were prepared as described previously (13). The MCG-B61, MCG-B69, and MCG-B71 cDNA probes were PCR fragments identified by CLONTECH PCR-Select cDNA Subtraction assay.[5] The KILLER/DR5 cDNA probe (GenBank #159553) was purchased from American Type Culture Collection. The following cDNA probes were purchased from Genome System, Inc. (St. Louis, MO): *BTG2* (GenBank #H86711), *14-3-3*σ (W79136), *PIG1* (W61024), *PIG2* (H18355), *PIG3* (N75824), *PIG4* (H45773), *PIG6* (R88591), *PIG8* (R42786), *PIG10* (R87338), *PIG11* (R54648), *PIG12* (AA149234), and *p85* (N21330).

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[4] The abbreviation used is: FACS, fluorescence-activated cell sorting.

[5] Manuscript in preparation.

Results

p73 Can Induce Cell Cycle Arrest and Apoptosis. Previously, we have established a number of H1299 non-small cell lung carcinoma cell lines that inducibly express p53 under a tetracycline-regulated promoter and showed that p53 can induce either cell cycle arrest or apoptosis (12). By using a similar approach, we generated several H1299 cell lines that inducibly express either p73α, p73β, or p73α292. p73α292(R292H) was previously shown to be defective in inducing growth suppression and apoptosis (2, 3). Two representative cell lines each that express either p73α, p73β, or p73α292 are shown in Fig. 1. p73α-22 expresses a high level of p73α when induced (+), whereas p73α-4 produces a moderate level of p73α (Fig. 1*A*). p73β-9 expresses a high level of p73β when induced (+), whereas p73β-26 produces a moderate level of p73β (Fig. 1*B*). p73α292-20 expresses a high level of p73α292 when induced (+), whereas p73α292-3 produces a low level of p73α292 (Fig. 1*C*). The amount of actin was also quantitated as an internal control (Fig. 1, *A–C, bottom*).

To characterize the activity of p73, growth rates of p73α-22, p73β-9, and p73α292-20 cell lines were determined. Dramatic differences were seen for wild-type p73, but not mutant p73α292-expressing cells, when the growth rates of uninduced and induced cells were compared (Fig. 2, *A*, *D*, *G*, and *J*). As shown in Fig. 2, *A* and *D*, cells that did not express p73 continued to grow (○, uninduced), whereas both the p73α- and p73β-expressing cells failed to multiply and started to die within 2 days on induction (▲, induced). These results are consistent with previous reports that p73α and p73β can cause growth suppression (2, 3). It should be mentioned that we also tested two other wild-type p73-expressing cell lines, p73α-4 and p73β-26. Results from these two cell lines were similar to that from p73α-22 and p73β-9 (data not shown). In contrast, the growth rates of p73α292-20 cells that inducibly express p73α292 were nearly identical under both the uninduced (□) and induced (◇) conditions (Fig. 2*G*). As control, the growth rates of H1299-24 cells that were similarly established but did not express any protein were nearly identical in the presence (□ +tet) or absence (◇ −tet) of tetracycline (Fig. 2*J*).

Next, we used FACS analysis to examine whether p73 can induce apoptosis and/or cell cycle arrest. Previously, we have shown that the percentage of cells containing a sub-G_1 content of DNA reflects the extent to which cells are undergoing apoptosis (12, 13). When p73 was not expressed, the percentage of p73α-22 and p73β-9 cells that had a sub-G_1 content of DNA were 2% and 1%, respectively (Fig. 2, *B* and *E*). However, when p73α or p73β was induced, the percentage of p73α-22 and p73β-9 cells that had a sub-G_1 content of DNA was increased to 20% and 44%, respectively (Fig. 2, *C* and *F*). These results indicate that the p73-expressing cells were undergoing apoptosis and confirm the previous report that p73 can induce apoptosis in the SAOS-2 osteosarcoma cell line (3). Furthermore, FACS analysis showed that the number of live cells containing S phase amounts of DNA was substantially decreased when p73α or p73β was expressed (compare Fig. 2, *B* with *C*, and Fig. 2, *E* with *F*). Quantitation by the Modfit program showed that the S phase cells were decreased from 35–12% for the p73α-22 cell line and from 34–20% for the p73β-9 cell line. In addition, the G_0-G_1 and G_2-M phase cells were increased on p73 expression. The G_0-G_1 phase cells were increased from 51–62% for the p73α-22 cell line (compare Fig. 2, *B* with *C*) and from 52–58% for the p73β-9 cell line (compare Fig. 2, *E* with *F*). The G_2-M phase cells were increased from 15–26% for the p73α-22 cell line (compare Fig. 2, *B* with *C*) and from 14–22% for the p73β-9 cell line (compare Fig. 2, *E* with *F*). These results indicated that the p73-expressing cells also underwent cell cycle arrest and the arrest occurred in both G_1 and G_2-M. It should be mentioned that we also tested p73α-4 and p73β-26 cell lines. Results from these two cell lines were similar to that from p73α-22 and p73β-9 (data not shown). In contrast, induction of mutant p73α292 or withdrawal of tetracycline had no effect on the cell cycle distribution of p73α292-20 cells (compare Fig. 2, *H* with *I*) and H1299-24 cells (compare Fig. 2, *K* with *L*), respectively.

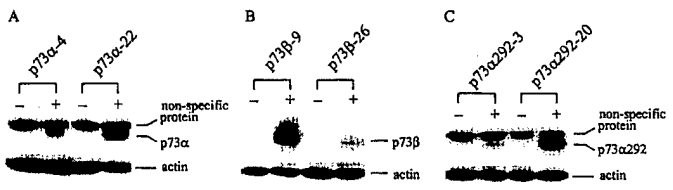

Fig. 1. Characterization of cell lines that inducibly express p73. *A*, levels of p73α (*top*) and actin (*bottom*) in p73α-4 and p73α-22 cell lines were assayed by Western blot analysis. Cell extracts were prepared from uninduced cells (−) and cells induced to express (+) p73α. The upper portion of the blot was probed with anti-hemagglutinin antibody 12CA5, and the bottom portion of the blot was probed with anti-actin polyclonal antibody. *B*, levels of p73β and actin in p73β-9 and p73β-26 cell lines. The experiments were performed similar to that in *A*. *C*, levels of p73α292 and actin in p73α292-3 and p73α292-20 cell lines. The experiments were performed similar to that in *A*.

Common and Distinctive Cellular Target Genes Activated by p53 and p73. Because the DNA binding domain in p73 is 63% identical to that in p53, it is reasonable to suggest that p73 can activate p53 target genes. We found that some p53 cellular target genes can be activated, but not others, by p73 (Fig. 3). In addition, most of the genes that can be activated by p73 were only weakly induced by p73 (Table 1). Interestingly, *14-3-3σ* and *PIG7* were induced by p73 to an extent that is substantially greater than by p53 (Table 1).

Previous reports have shown that the p21 promoter can be activated by p73 and that the level of p21 protein was increased in cells when exogenous p73 was expressed (2, 3), suggesting that the *p21* gene may also be a cellular target of p73. Consistent with these results, Northern blot analysis showed that p21 was induced by p73α and p73β in p73α-22 and p73β-9 cells, but not by mutant p73α292 in p73α292-20 cells (Fig. 3*A*). As expected, p21 was also induced by p53 in p53-3 cells when p53 was expressed (Fig. 3*A*). However, PhosphorImage quantitation showed that the level of p21 induced by p53 is at least three and six times higher than by p73β and p73α, respectively (Table 1). The results suggest that p53, p73α, and p73β may differ from each other in transcriptional activity, which is further supported by additional Northern blot analyses (see below).

Next, we determined whether p73 can regulate *MDM2*, *GADD45*, *BAX*, *BTG2*, 14-3-3σ, p85, and KILLER/DR5. We found that *MDM2*, an oncogene and a negative regulator of p53 (14), was significantly induced by p73β, albeit much less than by p53 (Fig. 3*B*). However, *MDM2* was induced little, if any, by p73α. *GADD45*, a DNA damage responsive gene involved in DNA repair (15), was slightly activated by p73 (Fig. 3*C*). BAX, an apoptosis activator that can be activated by p53 (8), was induced little, if any, by p73 (Fig. 3*D*). *BTG2*, a nerve growth factor responsive gene that can cause growth suppression (6), was weakly activated by p73 (Fig. 3*E*). Surprisingly, *14-3-3σ*, a gene that might mediate p53-dependent G_2-M arrest, was induced by p73α and p73β to an extent that is six and two times greater than by p53, respectively (Fig. 3*F*). p85 is a regulatory subunit of the signaling protein phosphatidyl-3-OH kinase that might be involved in the p53-dependent apoptotic response to oxidative stress (11). On H_2O_2 treatment, the level of p85 was increased in a p53-dependent manner (11). We found that p85 was induced 2–3-fold by either p53, p73α, or p73β, but not p73α292 (Fig. 3*G*; Table 1). *KILLER/DR5*, a death receptor gene that can be induced by genotoxic stress and p53 (9), was weakly induced by p53 and p73β but little, if any, by p73α (Fig. 3*H*; Table 1).

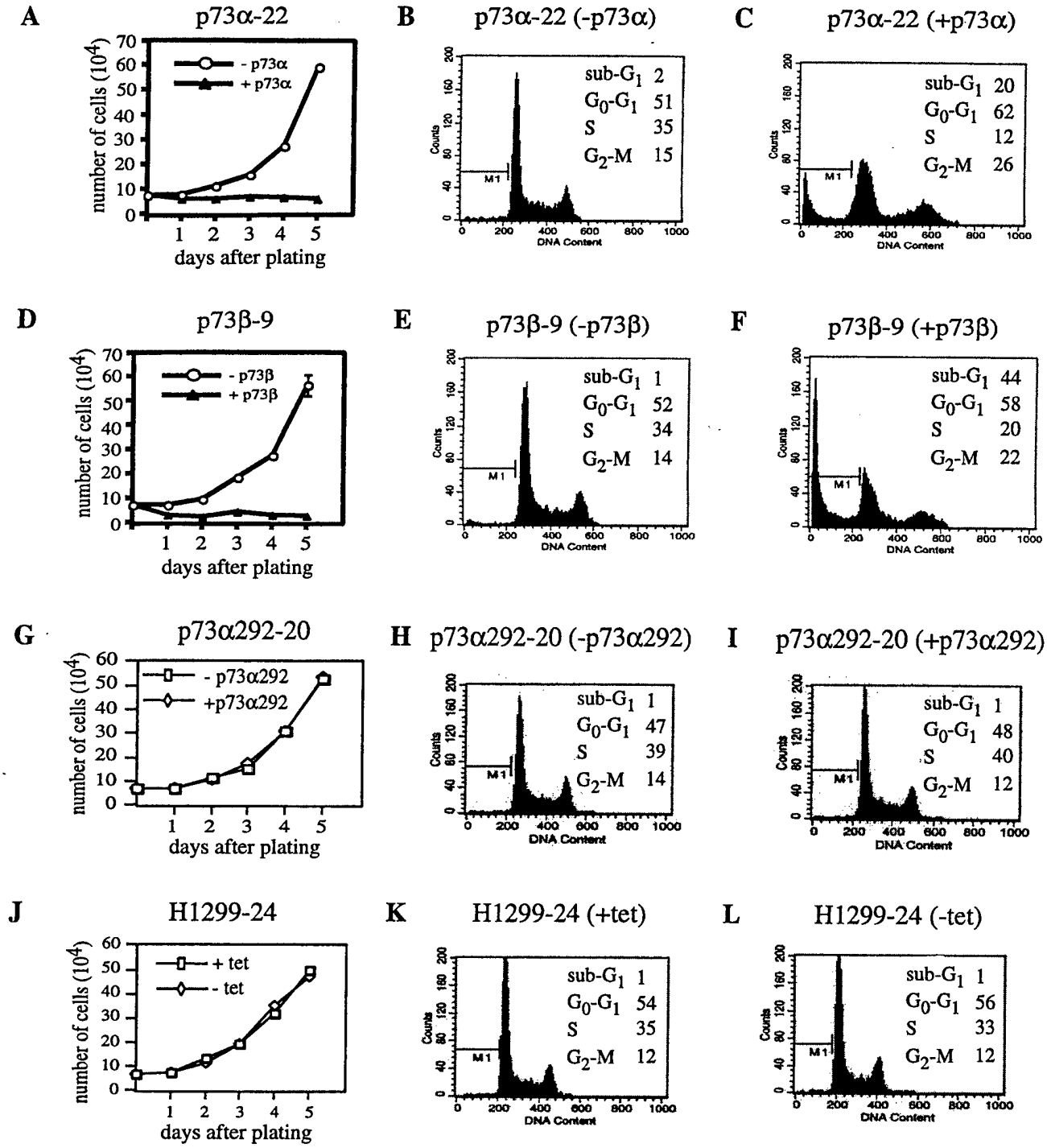

Fig. 2. p73 can induce both cell cycle arrest and apoptosis. *A*, *D*, *G*, and *J*, growth rates of p73-expressing and control cells under both the uninduced and induced conditions were measured as described in "Materials and Methods." *B*, *C*, *E*, *F*, *H*, *I*, *K*, and *L*, DNA contents of p73-expressing and control cells on day 3 under both the uninduced (*B*, *E*, *H*, and *K*) and induced (*C*, *F*, *I*, and *L*) conditions were quantitated by propidium iodide staining and FACS analysis as described in "Materials and Methods." M_1 represents cells that contain a sub-G_1 content of DNA. The percentage of apoptotic cells containing a sub-G_1 content of DNA under both the uninduced and induced conditions was quantitated using CellQuest program. The percentage of live cells in G_0-G_1, S, and G_2-M under both the uninduced and induced conditions was quantitated using ModFit program.

Several redox-related genes (PIGs), that were shown to be activated by p53 (10), were examined for p73 induction. We confirmed that *PIG2*, *PIG3*, *PIG6*, *PIG7*, *PIG8*, and *PIG11* were significantly induced by p53 in H1299 cells (Fig. 3, *I–N*). However, we found that *PIG3*, *PIG6*, and *PIG11* were not significantly induced by p73 (Fig. 3, *J*, *K*, and *N*) and *PIG2* was slightly induced by p73α but not by p73β (Fig. 3*I*). *PIG8* was induced by both p73α and p73β to a level slightly less than by p53 (Fig. 3*M*). *PIG7* was substantially induced by p73β to a level that is two times more than by p53 (Fig. 3*L*). We also found that *PIG10* and *PIG12* were not substantially induced by p53, and *PIG1* and *PIG4* were undetectable in H1299 cells (data not shown). Therefore, p73 induction of the *PIG1*, *PIG4*, *PIG10*, and *PIG12* genes was not analyzed.

We also examined three potential p53 target genes we recently

identified, *MCG-B61*, *MCG-B69*, and *MCG-B71*,[5] for p73 induction. *MCG-B61* was induced by p73β, but not by p73α (Fig. 3*O*). *MCG-B69* was significantly induced by p73 (Fig. 3*P*). However, *MCG-B71* was not induced by p73 (Fig. 3*Q*).

Discussion

The cell lines described above provide several novel observations. Similar to p53, p73 can induce cell cycle arrest and apoptosis in H1299 cells. We also demonstrate that although p73 is homologous to p53 in the activation and DNA binding domains, p73 differentially activate some but not all p53 cellular target genes.

Our data provide evidence that p73 can induce cell cycle arrest. FACS analysis showed that p73-dependent arrest occurs in both G_1 and G_2-M, similar to that by p53. Although p73 can induce p21, the level of p21 induced by p73α and p73β in p73α-22 and p73β-9 cells is six and three times lower than that by p53 in p53-3 cells, respectively (Fig. 3*A*). Two p53 target genes that can cause growth suppression and may be involved in cell cycle arrest, *i.e.*, *GADD45* (15) and *BTG2* (6), are only weakly activated by p73. Therefore, it remains to determine whether the level of p21 induced by p73 is sufficient to cause cell cycle arrest and whether other cellular genes may also be involved in p73-dependent cell cycle arrest.

It has been shown that p53-dependent G_2-M arrest is at least, in part, mediated by up-regulation of *14-3-3*σ (5). The gene product interacts with cdc25 phosphatase to block activation of cyclin B-dependent cdc2 kinase, which is required for initiation of mitosis, leading to arrest in G_2-M (16). We found that p73 can also activate *14-3-3*σ (Fig. 3*F*), suggesting that a signaling pathway to induce arrest in G_2-M is conserved between p53 and p73. Since activation of *14-3-3*σ by p73 is 3–6 times higher than by p53, it suggests that *14-3-3*σ may be a bona fide cellular target of p73, even though it was originally identified as a potential p53 target gene.

Although both p53 and p73 can induce apoptosis, the signaling pathway leading to apoptosis may differ from each other on the basis of the differential ability of p73 to activate some p53 cellular target genes. *BAX* and several redox-related genes (*PIG2*, *PIG3*, *PIG6*, and *PIG11*), that may be involved in mediating p53-dependent apoptosis (8, 10), were not significantly induced by p73 (Fig. 3). *KILLER/DR5*, a gene that may mediate p53- and DNA damage-induced apoptosis (9), was weakly induced by p53 and p73β but not p73α in H1299 cells (Fig. 3). Since we have shown that p73α is nearly as active as p73β in inducing apoptosis (Fig. 2), the results suggest that *KILLER/DR5* may not be involved in p73-dependent apoptosis unless an apoptotic signaling pathway for p73α is different from that for p73β. Although *PIG7*, *PIG8*, and *p85* were significantly induced by p73, the activity of *PIG7* and *PIG8* in apoptosis is still unknown, and the role of *p85* in apoptosis seems to be restricted to cellular response to oxidative stress (11). Because the transactivation-deficient p73α292 is incapable of inducing apoptosis (Refs. 2 and 3; present study), we hypothesize that a distinctive group of cellular genes that can be activated by p73 may be responsible for mediating apoptosis. Therefore, identification of such p73 target genes is necessary to understand the mechanism of p73-dependent apoptosis.

MDM2, an oncogene that negatively regulates p53 activity (14), was weakly induced by p73β but not p73α (Fig. 3*B*). It has been shown that a physical interaction between p53 and *MDM2* enhances degradation of p53 through the ubiquitination pathway (17, 18) as well as conceals the activation domain of p53 to regulate transcription (19). Although it is not clear whether p73 physically interacts with MDM2, failure to activate MDM2 by p73α suggests that the activity of p73α is not subject to regulation by MDM2. Thus, in addition to a previous report that p73 is not responsive to DNA damage (2), it is also regulated differently from p53 by MDM2.

Table 1 *Induction of cellular targets by p53 and p73*

	Fold increase in relative mRNA[a]			
	p53	p73α	p73β	p73α292
p21	17.5	3.2	6.4	1.4
MDM2	14.8	1.4	6.5	0.9
GADD45	4.3	1.5	3.6	1.6
BAX	3.6	0.8	1.2	1.0
BTG2	38.9	4.2	10.1	0.8
14-3-3σ	3.0	17	6.5	ND[b]
p85	2.8	2.0	2.5	1
KILLER/DR5	3	1.2	3	0.7
PIG2	5.0	1.5	0.7	1.0
PIG3	21	2	5	0.5
PIG6	9.9	ND	ND	ND
PIG7	2.4	3.0	5.0	0.9
PIG8	4.7	3	3	0.8
PIG11	15.4	ND	ND	ND
MCG-B61	3.3	0.9	3.0	0.7
MCG-B69	4.3	3.0	3.5	0.7
MCG-B71	3.5	0.7	1.0	1.0

[a] Fold = mRNA(+p53 or +p73)/mRNA(−p53 or −p73).
[b] ND, not done.

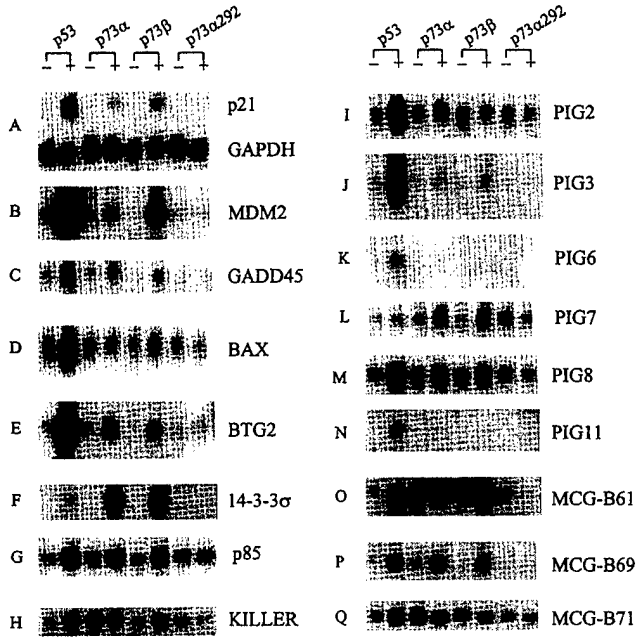

Fig. 3. p73 differentially activates some but not all of the *p53* cellular target genes. Northern blots were prepared using 10 μg of total RNA isolated from p53-3, p73α-22, p73β-9, or p73α292-20 cells under both the uninduced (−) and induced (+) conditions. The blots were probed with cDNAs corresponding to the genes as indicated on the right side of the blots.

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Appendix 12

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p63α and ΔNp63α can induce cell cycle arrest and apoptosis and differentially regulate p53 target genes

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The p53 tumor suppressor protein plays a critical role in the regulation of the cell cycle and apoptosis. The importance of p53's functions is underscored by the high incidence of p53 mutations in human cancers. Recently, two p53-related proteins, p73 and p63, were identified as members of the *p53* gene family. Multiple isoforms of p73 have been found, including ΔN variants in which the N-termini are truncated. p63 is expressed as three major forms, p63α, p63β and p63γ, each of which differ in their C-termini. All three forms can be alternatively transcribed from a cryptic promoter located within intron 3, producing ΔNp63α, ΔNp63β and ΔNp63γ. The high degree of similarity of p73 and p63 to evolutionarily conserved regions of p53 suggests that these proteins play an important and potentially redundant role in regulating cell cycle arrest and apoptosis. Here we describe the characterization of cell lines generated to inducibly express p63α and ΔNp63α. We have found that p63α and ΔNp63α can differentially regulate endogenous p53 target genes and induce cell cycle arrest and apoptosis. Deletion of the N-terminal 26 amino acids of ΔNp63α abolished its ability to transactivate p53 target genes and induce cell cycle arrest and apoptosis. This indicates that a putative transactivation domain exists within the N-terminus of the ΔN variants of p63. Furthermore, the differential regulation of p53 target genes by p63α and ΔNp63α suggests that p63 and p53 utilize both similar and different signaling pathways to execute their cellular functions. *Oncogene* (2001) **20,** 3193–3205.

Keywords: apoptosis; cell cycle arrest; p63; p53; p73

Introduction

More than 50% of all human cancers contain mutations in the *p53* tumor suppressor gene (Hollstein *et al.*, 1991, 1994). In response to cellular stresses such as DNA damage and hypoxia, p53 is activated and can mediate cell cycle arrest and apoptosis via the upregulation of numerous target genes (for reviews, see Agarwal *et al.*, 1998; Levine, 1997; Prives and Hall, 1999). $p21^{WAF1}$, a potent inducer of cell cycle arrest (el-Deiry *et al.*, 1993; Harper *et al.*, 1993), and Bax (Miyashita and Reed, 1995) and MCG10 (Zhu and Chen, 2000), initiators of the apoptotic cascade, are p53 target genes. p53-null mice, as well as humans containing germline mutations at the *p53* locus, are susceptible to spontaneous tumors (Donehower *et al.*, 1992; Evans and Lozano, 1997). The observation that p53-null mice maintain normal embryonic development (Donehower *et al.*, 1992), however, has raised the possibility that there exists a family of closely related proteins with overlapping functions. Recently, two new genes, *TP73* and *TRP63*, have been found to encode several p73 and p63 proteins, respectively, with structures and functions related to p53 (for reviews, see Chen, 1999; Kaelin, 1999; Levrero *et al.*, 2000; Lohrum and Vousden, 2000).

The first p53 homolog described was p73 (Kaghad *et al.*, 1997). There are at least six isoforms of p73, most of which differ in the C-terminal region and have varied transcriptional activites (De Laurenzi *et al.*, 1998, 1999; Kaghad *et al.*, 1997; Ueda *et al.*, 1999; Zaika *et al.*, 1999). p73 shares considerable sequence identity with p53, reaching 63% within the DNA-binding domain and including highly conserved DNA contact residues frequently mutated in tumors (Kaghad *et al.*, 1997). Homology between p73 and p53 also exists within the N-terminal transactivation domains (29% identity) and the oligomerization domains (38% identity) (Kaghad *et al.*, 1997). p73 can bind to the p53 consensus DNA-binding motif and activate a number of p53-regulated genes, including $p21^{WAF1}$ (Jost *et al.*, 1997; Zhu *et al.*, 1998a). p73 can also induce cell cycle arrest and apoptosis (Jost *et al.*, 1997; Zhu *et al.*, 1998a). Accumulation and phosphorylation of p73 are seen in response to DNA damage; however, different DNA-damage inducers appear to have varied affects on p73 activation (Agami *et al.*, 1999; Gong *et al.*, 1999; Levrero *et al.*, 1999; Shaul, 2000; Yuan *et al.*, 1999). The differential regulation of p53 target genes by p73 indicates that although the activities of p53 and p73 overlap these two proteins also maintain separate and unique functions within a cell (Zhu *et al.*, 1998a). p73 proteins that lack the N-terminal transactivation domain have recently been found (Yang *et al.*, 2000). These ΔNp73 proteins, although capable of binding p53 response elements, have been reported to be deficient in transactivating a promoter that contains a

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p53 response element (Yang *et al.*, 2000). Furthermore, ΔNp73 has been implicated as a dominant negative isoform of p73 (Yang *et al.*, 2000).

More recently, a second p53 homolog, named p63, was described (Osada *et al.*, 1998; Schmale and Bamberger, 1997; Senoo *et al.*, 1998; Trink *et al.*, 1998; Yang *et al.*, 1998). The *TRP63* locus expresses three major forms, p63α, p63β and p63γ, which, like p73, differ in the C-terminal region (Yang *et al.*, 1998). Each of these forms can be alternatively transcribed from a cryptic promoter located within intron 3, producing ΔNp63α, ΔNp63β and ΔNp63γ (Yang *et al.*, 1998). Additionally, all six of these forms can exist as splicing variants in which four amino acids are deleted from exon 9 (Yang *et al.*, 1998). p63 contains regions of homology to p53 and shares 60% sequence identity with the DNA-binding domain, 22% identity with the N-terminal transactivation domain and 37% identity with the oligomerization domain of p53 (Osada *et al.*, 1998). p63γ has been shown to transactivate reporter constructs containing p53 response elements derived from p53 target genes (Osada *et al.*, 1998; Shimada *et al.*, 1999; Yang *et al.*, 1998), as well as regulate endogenous *p21^{WAF1}* expression (Osada *et al.*, 1998). In addition, overexpression of p63γ can suppress growth and induce apoptosis (Osada *et al.*, 1998; Yang *et al.*, 1998). In contrast to p53 and p73, p63 expression has been reported to be suppressed under conditions of DNA damage (Hall *et al.*, 2000; Liefer *et al.*, 2000). P63 knockout mice exhibit major limb, craniofacial, and epithelial defects, indicating that p63 is critical for proper epithelial development (Mills *et al.*, 1999; Yang *et al.*, 1999). The truncated ΔNp63 isoforms are thought to be transcriptionally inactive due to the lack of an acidic N-terminus corresponding to the transactivation domain of p53 (Yang *et al.*, 1998). the ΔNp63α and ΔNp63γ variants have been reported to be functionally inactive in transactivating a promoter that contains a p53 response element and in inducing cell death (Yang *et al.*, 1998). Furthermore, the ΔNp63α and ΔNp63γ isoforms have been shown to exhibit dominant-negative effects toward both p53 and p63 (Yang *et al.*, 1998), and ΔNp63 variants are thought to be oncogenic (Hibi *et al.*, 2000). However, many questions regarding the transactivation activities of the p63 isoforms have yet to be answered. Although p63γ and p63α share identical N-terminal transactivation domains, p63γ exhibits strong transactivation of promoters that contain p53 response elements and is a potent inducer of cell death, whereas p63α has no detectable transactivation activity (Yang *et al.*, 1998).

In this study, we generated stable p53-null, H1299 cell lines that inducibly express p63α and ΔNp63α under a tetracycline-regulated promoter. In contrast to previous reports, we have found that these p63 isoforms are transcriptionally active and differentially regulate endogenous p53 target genes. In addition, we found that p63α and ΔNp63α are capable of inducing cell cycle arrest and apoptosis. These findings indicate that a putative transactivation domain exists within the N-terminus of the ΔN variants of p63. Subsequent deletion of the N-terminal 26 amino acids of ΔNp63α abrogated the ability of this protein to regulate p53 target genes and induce cell cycle arrest and apoptosis. Furthermore, the differential regulation of p53 and p73 target genes by p63 indicates that although the activities of p53-family members overlap, they also maintain separate and unique cellular functions.

Results

Generation of p63α- and ΔNp63α-expressing cell lines

Previously, several studies (Osada *et al.*, 1998; Shimada *et al.*, 1999; Yang *et al.*, 1998) showed that p63γ can induce apoptosis and p21^{WAF1}, a cyclin-dependent kinase inhibitor that is primarily responsible for p53-dependent cell cycle arrest (el-Deiry *et al.*, 1993; Harper *et al.*, 1993). However, p63α and the ΔN variants of p63 have been shown to be incapable of inducing apoptosis or p21^{WAF1} (Yang *et al.*, 1998). To further determine the activity of p63α and ΔNp63α in the regulation of the cell cycle, we have generated H1299 non-small cell lung carcinoma cell lines that inducibly express these proteins under a tetracycline-regulated promoter. Two representative cell lines for both p63α and ΔNp63α are shown in Figure 1. ΔNp63α-11 and ΔNp63α-22 expressed high levels of ΔNp63α protein, whereas p63α-11 and p63α-14 expressed moderate levels of p63α protein when induced (Figure 1, upper panel). Interestingly, we found that p63α and ΔNp63α were able to induce p21^{WAF1} (Figure 1, middle panel). The levels of actin were determined as a loading control (Figure 1, lower panel).

p63 and p73 isoforms have been suggested to form homotypic and heterotypic complexes (Davison *et al.*, 1999; Yang *et al.*, 1998) that could potentially influence activity of the inducible p63 in the above cell lines. To

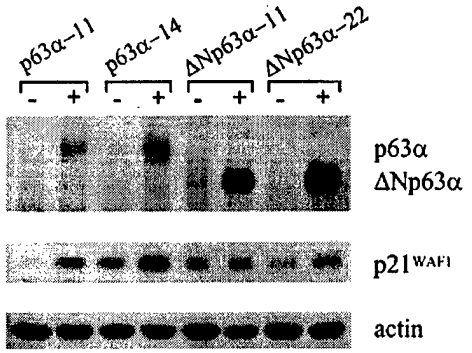

Figure 1 Characterization of cell lines that inducibly express p63. Levels of p63α and ΔNp63α (upper panel), p21^{WAF1} (middle panel), and actin (lower panel) in p63α-11, p63α-14, ΔNp63α-11, and ΔNp63α-22 cell lines were assayed by Western blot analysis. Cell extracts were prepared from uninduced cells (−) and cells induced to express p63α or ΔNp63α (+) for 24 h. The upper portion of the blot was probed with anti-myc antibody 9E10.2, the middle portion of the blot was probed with anti-p21 antibody C19, and the lower portion of the blot was probed with anti-actin polyclonal antibody

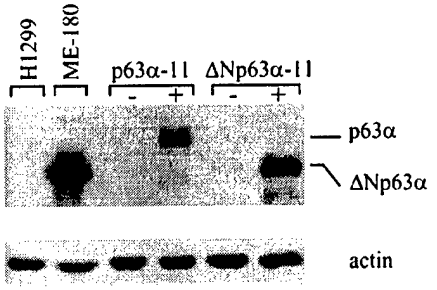

Figure 2 H1299 cell line has undetectable levels of endogenous p63. Levels of p63 (upper panel) and actin (lower panel) in H1299, ME-180, p63α-11, and ΔNp63α-11 cell lines were assayed by Western blot analysis. Cell extracts were prepared from H1299, ME-180, and p63α-11 and ΔNp63α-11 that were uninduced (−) and induced (+) to express p63 for 24 h. The upper portion of the blot was probed with anti-p63 antibody Ab-1, and the lower portion was probed with anti-actin polyclonal antibody

examine this possibility, we analysed the endogenous levels of these proteins in the parental H1299 cell line. As shown in Figure 2, p63 isoforms were not detectable in H1299 cells; however, as previously shown (Yang *et al.*, 1998), ΔNp63α was detectable in the cervical carcinoma cell line ME-180 (Figure 2). p63 was detectable in p63α-11 and ΔNp63α-11 cell lines only when induced to express their respective p63 proteins, and ΔNp63α was found at levels less than that in ME-180 cells (Figure 2, upper panel). The levels of actin were determined as a loading control (Figure 2, lower panel). Only the ΔNp63α isoform of p63 was detected in ME-180 cells, which is consistent with previous reports of predominant expression of ΔN variants (Crook *et al.*, 2000; De Laurenzi *et al.*, 2000; Hall *et al.*, 2000; Yang *et al.*, 1998). Additionally, p73 was undetectable in the H1299 cell line (data not shown).

p63α and ΔNp63α can induce cell cycle arrest and apoptosis

To characterize the activity of p63, we analysed the effect of p63α and ΔNp63α expression on the growth rates of these cell lines. As shown in Figure 3, expression of p63α and ΔNp63α suppressed cell proliferation. The uninduced cells (□) grew normally, whereas growth of p63α- and ΔNp63α-expressing cells (◆) was severely diminished or nearly abolished up to 5 days after plating (Figure 3a–d). The growth rates of parental H1299 cells under induced and uninduced conditions were nearly identical (data not shown).

To determine whether the anti-proliferative effect of p63α and ΔNp63α was due to cell cycle arrest, apoptosis, or both, we used FACS analysis to examine the DNA content of p63-expressing cells. When p63 was not expressed, all four cell lines maintained similar cell cycle phase distributions of DNA content (Figure 4a,e,i,m). In contrast, when p63 was expressed, an increase in G_1 DNA content and a decrease in S phase DNA content were detected (Figure 4b,f,j,n). No significant changes were observed in G_2-M phase DNA content. The increase in the number of G_1 phase cells indicates that p63α and ΔNp63α can induce G_1-arrest in H1299 cells. Additionally, we found an increased number of cells with a sub-G_1 DNA content, suggesting that p63 can induce apoptosis (Figure 4, compare b with a, f with e, and n with m). To examine this further, we performed an annexin V staining assay. We found that p63 expression resulted in an increase in total annexin V positive cells (sum of the upper and lower right quadrants) (Figure 4). The total percentage of annexin V positive cells increased from 4–20% (Figure 4, compare c with d) and from 4–16% (Figure 4, compare g with h) for cells lines p63α-11 and p63α-14, respectively. Annexin V positive cells increased from 8–13% (Figure 4, compare k with l) and from 5–17% (Figure 4, compare o with p) for cell lines ΔNp63α-11 and ΔNp63α-22, respectively. These results indicate that in addition to arresting cells in G_1 phase, p63α and ΔNp63α are capable of inducing apoptosis.

Next, we performed a Trypan blue dye exclusion assay. As shown in Figure 5a, cells expressing p63α and ΔNp63α contained a consistently higher percentage of Trypan blue positive cells compared to those not expressing p63. This supports the above results and suggests that p63 can induce apoptosis. Additionally, we examined the effect of p63 expression on mitochondrial membrane potential. This assay utilizes fluorescence to distinguish between healthy and apoptotic cells. Induced and uninduced cells were stained with Mitosensor, a cationic dye. In healthy cells, the dye aggregates in the mitochondria and fluoresces an intense red. Upon induction of apoptosis, changes in the cell's mitchondrial membrane potential prevent aggregation of the dye, resulting in a decrease in the mean fluorescence. As shown in Figure 5b, expression of p63α and ΔNp63α resulted in a decrease in relative mean fluorescence. p53-expressing cells were assayed as a positive control. The relative mean fluorescence was similarly decreased in cells expressing p53, p63α, or ΔNp63α.

Differential regulation of p53 and p73 targets by p63

The high degree of similarity between p53, p73, and p63 DNA-binding domains, as well as the considerable similarity between the transactivation and oligomerization domains, led us to examine the ability of p63 to activate p53 and p73 target genes. We have found that some p53 and p73 target genes can be activated by p63α, ΔNp63α, or both, while others were not induced (Figure 6 and data not shown). However, most genes that could be activated by p63 were only weakly induced (Figure 6).

Previous reports have shown that p63γ can transactivate p53 response elements within the $p21^{WAF1}$ promoter (Osada *et al.*, 1998; Shimada *et al.*, 1999). and our data showed that endogenous $p21^{WAF1}$ transcript was induced when p63α was expressed, and ΔNp63α was able to weakly activate $p21^{WAF1}$ (Figure 6a). These results are consistent with the induction of

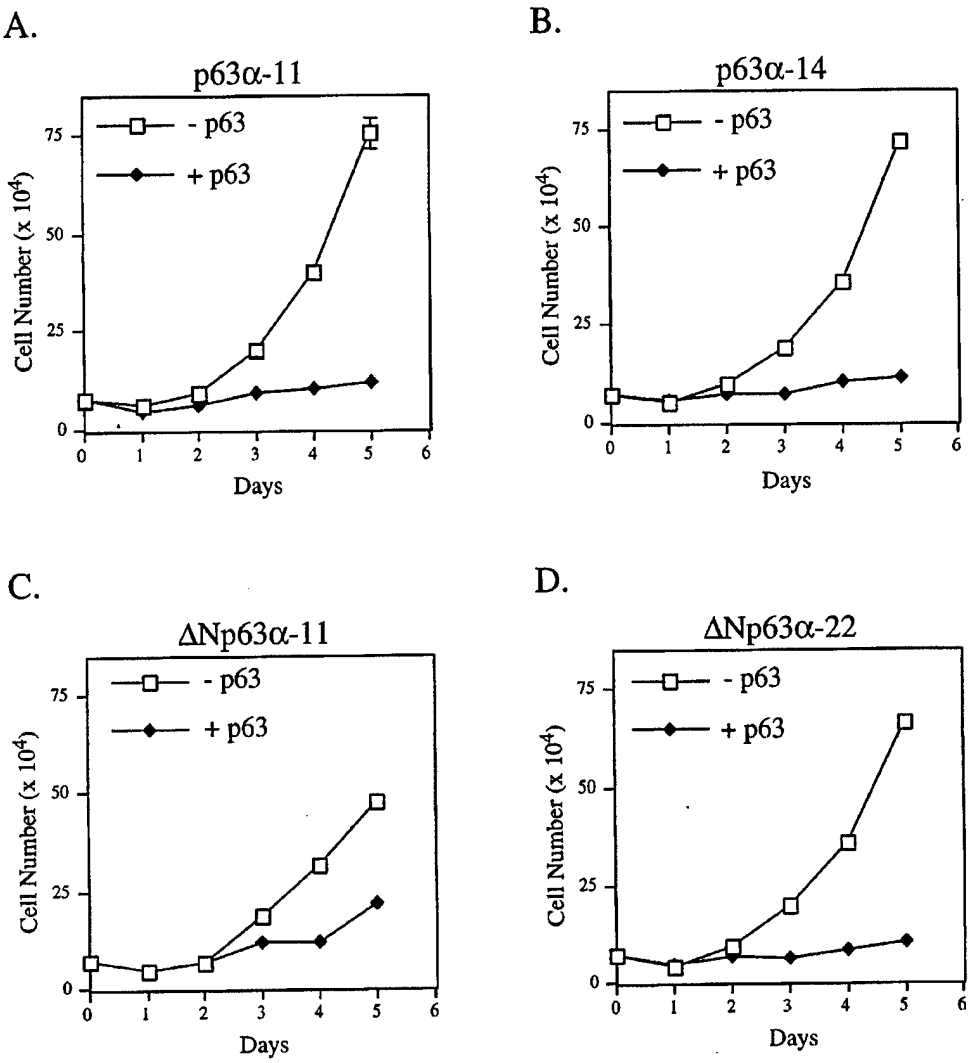

Figure 3 Both p63α and ΔNp63α negatively regulate cell proliferation. Growth rates of p63α-11 (**a**), p63α-14 (**b**), ΔNp63α-11 (**c**), and ΔNp63α-22 (**d**) under both uninduced (□) and induced (◆) conditions were measured as described in Materials and methods

$p21^{WAF1}$ protein by p63α and ΔNp63α (see Figure 1), as well as with the previous finding that endogenous $p21^{WAF1}$ can be induced by p63γ in a transient transfection assay (Osada *et al.*, 1998).

Next we examined whether p63 can regulate the *MDM2*, *GADD45*, *KILLER/DR5*, *BAX*, *p85*, *BTG2*, and *14-3-3σ* genes. We found that MDM2, a negative regulator of p53 (Wu *et al.*, 1993), was weakly induced by p63α but not by ΔNp63α (Figure 6b). Interestingly, the DNA damage responsive gene *GADD45* (Kastan *et al.*, 1992) was not activated by p63α but was highly induced by ΔNp63α (Figure 6c). *KILLER/DR5*, a death receptor gene induced by genotoxic stress (Wu *et al.*, 1997), was weakly induced by ΔNp63α but not by p63α (Figure 6d). *BAX*, an initiator of the apoptotic cascade (Miyashita and Reed, 1995), and *p85*, a signal transducer in the cellular response to oxidative stress (Yin *et al.*, 1998), were not induced by either p63α or ΔNp63α (data not shown). *BTG2* and *14-3-3σ*, two p53-inducible genes involved in cell cycle arrest in G_2 (Hermeking *et al.*, 1997; Rouault *et al.*, 1996), were not induced by either p63 isoform (data not shown).

We further examined whether p63 can regulate several redox-related p53-inducible genes (*PIG*s) (Polyak *et al.*, 1997). We found that *PIG3* was strongly induced by p63α (Figure 6e), and *PIG8* was weakly induced by p63α (Figure 6f). *PIG6*, *PIG7*, and *PIG11* were not induced by p63α or ΔNp63α (data not shown). We did not analyse p63 induction of *PIG10* and *PIG12*, which are not induced by p53 in H1299 cells (data not shown), and *PIG1* and *PIG4*, which are undetectable in H1299 cells (data not shown).

To further examine the regulation of p53 target genes by p63α and ΔNp63α, we performed luciferase reporter assays. The promoter of the p53-target gene

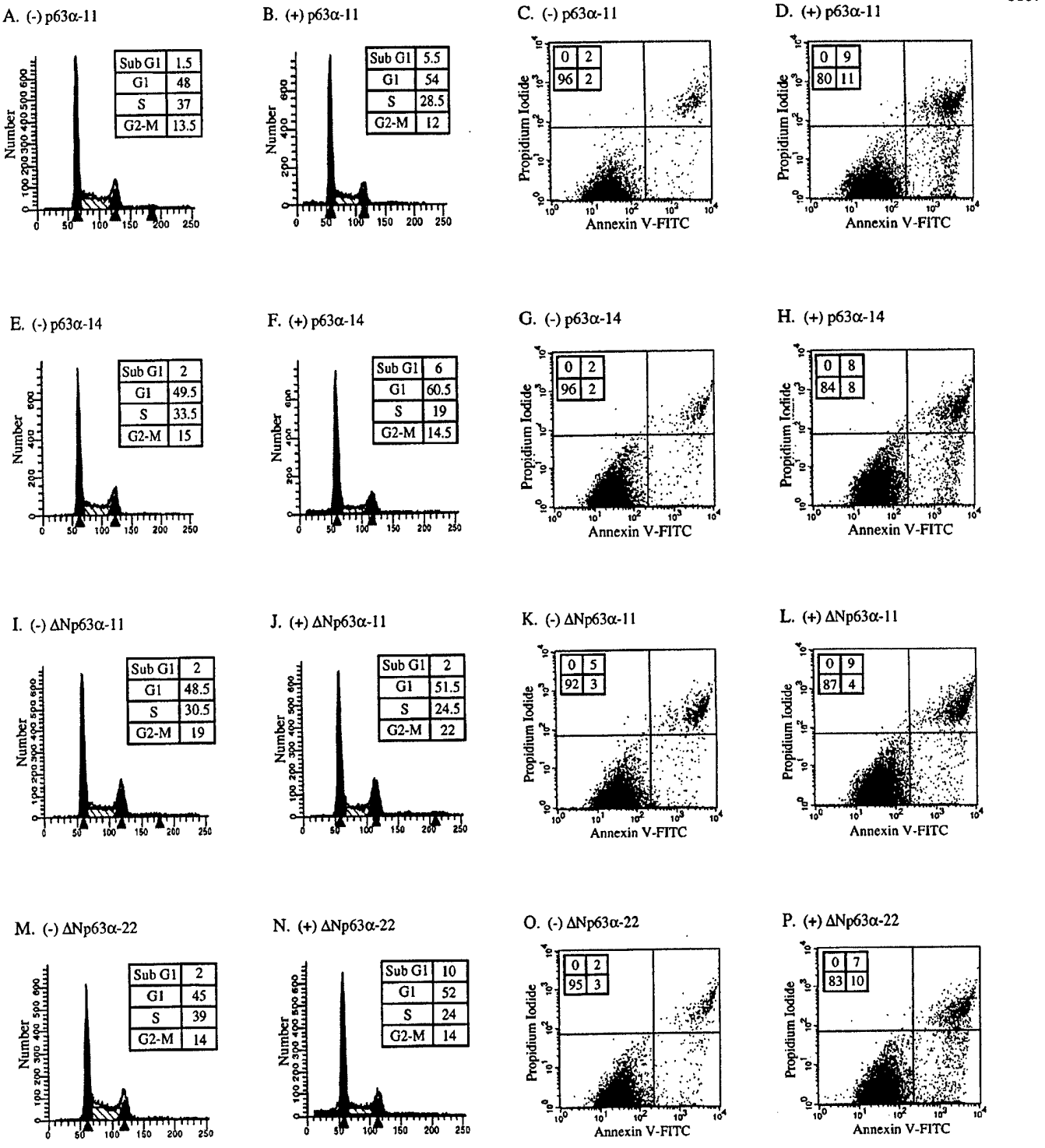

Figure 4 p63α and ΔNp63α are capable of inducing cell cycle arrest and apoptosis. (**a**,**b**,**e**,**f**,**i**,**j**,**m**, and **n**) DNA content was quantified by propidium iodide staining of fixed cells from p63-expressing cell lines that were uninduced (−) or induced (+) to express p63α or ΔNp63α for 3 days. (**c**,**d**,**g**,**h**,**k**,**l**,**o**, and **p**) Apoptotic cells were quantified by propidium iodide-annexin V staining of cells that were uninduced (−) or induced (+) to express p63α or ΔNp63α for 3 days

p21^{WAF1} was cloned upstream of a luciferase reporter gene, producing a *p21^{WAF1}* reporter construct (Chinery *et al*., 1997). The p53-response element located within the *EphA2* promoter (Dohn *et al*., 2001, unpublished results) was cloned upstream of the c-*fos* minimal promoter and a firefly luciferase reporter gene

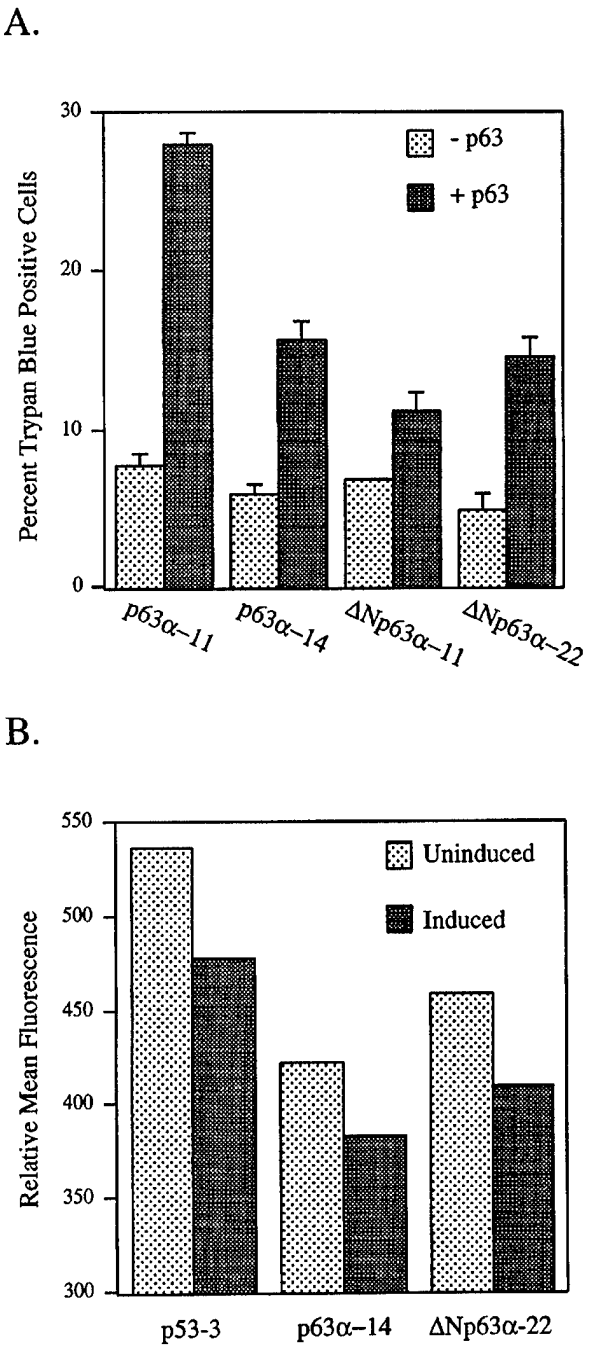

Figure 5 p63α and ΔNp63α are capable of inducing cell death. (**a**) Live and dead cells from p63-expressing cell lines that were uninduced (−) or induced (+) to express p63α or ΔNp63α were stained with Trypan blue dye solution. The percentage of dead cells (blue cells/total cells) was determined 3 days post induction. (**b**) Cells induced or uninduced to express wild-type p53 (p53-3), p63α (P63α-14), or ΔNp63α (ΔNp63α-22) for 3 days were stained with a mitochondrial staining reagent as described in Materials and methods. A decrease in the relative fluorescence is indicative of an increase in apoptosis

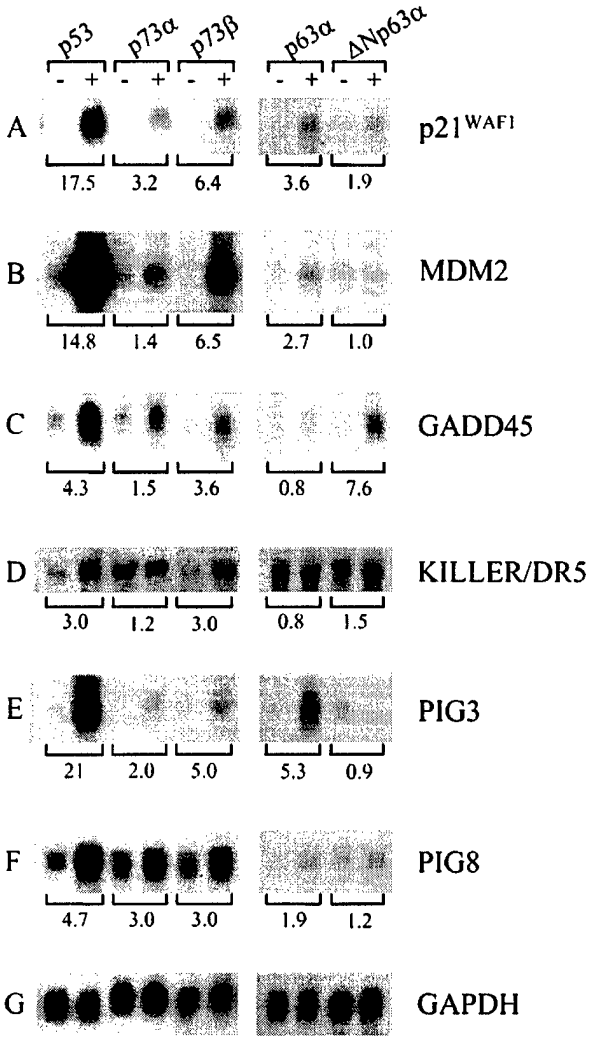

Figure 6 p63α and ΔNp63α differentially activate some but not all of the selected p53 and p73 target genes. Northern blots were prepared using 10 μg of total RNA isolated from p53-3, p73α-22, p73β-9, p63α-14, or ΔNp63α-22 cells under both the uninduced (−) and induced (+) conditions for 24 h. The blots were probed with cDNAs corresponding to the genes as indicated to the right of each blot, and mRNA levels were normalized to *GAPDH* (**g**). The fold increase in mRNA expression is shown below each blot

(Johansen and Prywes, 1994). These resulting reporter constructs were cotransfected into H1299 cells with either a pcDNA3 control vector or a vector expressing p53, p63α, or ΔNp63α. We found that the luciferase activity of the *p21*^{WAF1} and *EphaA2* reporter constructs was significantly increased when cotransfected with p53 (Figure 7). Similarly, p63α was able to transactivate to *p21*^{WAF1} reporter construct nearly fivefold greater than a pcDNA3 control vector, and the *EphA2* reporter construct was strongly transactivated by p63α (Figure 7). We found that similar to the Northern blot results in Figure 6a, ΔNp63α weakly transactivated the *p21*^{WAF1} reporter construct (Figure 7). Furthermore, ΔNp63α increased the luciferase activity of the *EphA2* reporter construct nearly fourfold greater than a pcDNA3 control vector (Figure 7).

The N-terminal 26 amino acids are necessary for ΔNp63α activity

Although the ΔNp63α isoform lacks the N-terminal transactivation domain (Yang *et al.*, 1998), it retains the ability to regulate some p53 target genes and induce cell cycle arrest and apoptosis (see Figures 4–7). This indicates that a potential second transactivation domain exists within this p63 isoform. In an attempt to further define this domain, we generated a ΔNp63α N-terminal deletion mutant (Table 1). This protein, called ΔΔNp63α, lacks the N-terminal 26 amino acids of ΔNp63α and was myc-tagged at the N-terminus, similarly to p63α and ΔNp63α. A representative cell line, which inducibly expresses ΔΔNp63α under a tetracycline-regulated promoter, is shown in Figure 8a. ΔΔNp63α protein was expressed (+) in the ΔΔNp63α-16 cell line (Figure 8a, upper panel) at a level comparable to the p63α-expressing cell lines (data not shown). To determine whether ΔΔNp63α has transcriptional activity, we analysed $p21^{WAF1}$ levels in this cell line. We found that unlike p63α and ΔNp63α, ΔΔp63α was incapable of inducing $p21^{WAF1}$ expression (Figure 8a, middle panel).

To further characterize the activity of ΔΔNp63α, we examined its effect on the growth rate of this cell line. As shown in Figure 8b, cells that were induced to express ΔΔNp63α(◆) grew at a rate similar to that of uninduced cells (□). In addition, there was no significant increase in the number of G_1 or sub-G_1 cells when ΔΔNp63α was induced (Figure 8d) as compared to that of uninduced cells (Figure 8c). Consistent with the DNA histogram analysis, the annexin V staining assay showed that the number of annexin V positive cells was not increased in cells induced to express ΔΔNp63α (data not shown). Furthermore, there was no significant difference in the number of Trypan blue stained cells when induced (+) to express ΔΔNp63α (Figure 8e). Since the DNA-damage responsive gene *GADD45* is strongly induced by ΔNp63α (see Figure 6c), we determined whether *GADD45* is regulated by ΔΔNp63α. We found that unlike ΔNp63α, the ΔΔNp63α deletion mutant was incapable of inducing *GADD45* (Figure 9). These results suggest that ΔΔNp63α is inactive in transactivation and is inert in inducing apoptosis and cell cycle arrest. The activities of p63α, ΔNp63α, and ΔΔNp63α are summarized in Table 1.

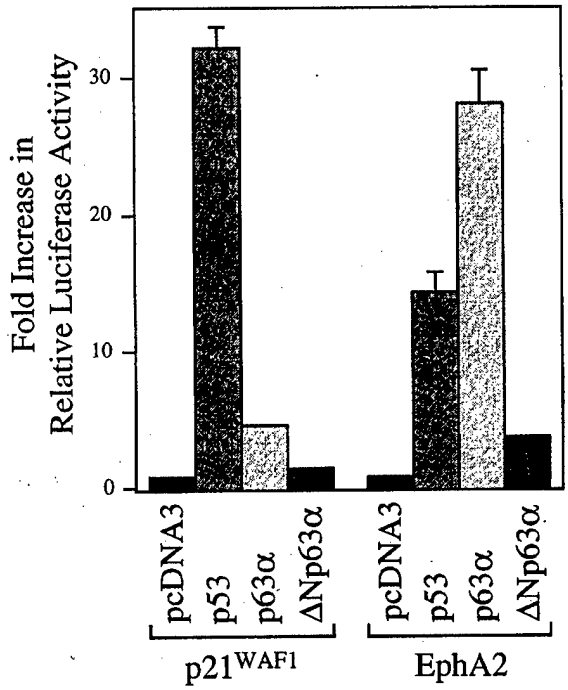

Figure 7 p63α and ΔNp63α transactivate p53-reporter genes. The *$p21^{WAF1}$* promoter that contains two p53 response elements was cloned upstream of a firefly luciferase reporter gene, and a p53 response element found in the *EphA2* promoter was cloned upstream of the c-*fos* minimal promoter and a firefly luciferase reporter gene. The resulting reporter constructs were cotransfected into H1299 cells with either pcDNA3 empty vector control or a vector expressing p53, p63α, or ΔNp63α. The firefly luciferase activity was measured from three different experiments and normalized with constitutive expression of Renilla luciferase

Table 1 p63 domains and activity

	Domain					Activity			
	AD1[1]	AD2[1]	PRD[1]	DBD[1]	TD[1]	p21[2]	arrest[3]	apoptosis[4]	GADD45[5]
p63α						+++	+++	+++	–
ΔNp63α[6]						+	+++	++	+++
ΔΔNp63α						–	–	–	–

[1]AD1, activation domain 1 within residues 1-59
AD2, potential activation domain 2 within residues 60-86
PRD, proline-rich domain within residues 67-127
DBD, DNA-binding domain within residues 142-321
TD, tetramerization domain within residues 353-397
[2]The ability of p63 to induce p21 as measured by Western and Northern blot analyses
[3]The ability of p63 to induce cell cycle arrest as measured by DNA histogram analysis
[4]The ability of p63 to induce apoptosis as measured by trypan dye exclusion, annexin V staining, and DNA histogram analysis
[5]The ability of p63 to induce GADD45 as measured by Northern blot analysis
[6]ΔNp63 isoforms contain 14 unique amino acids at the N-terminus

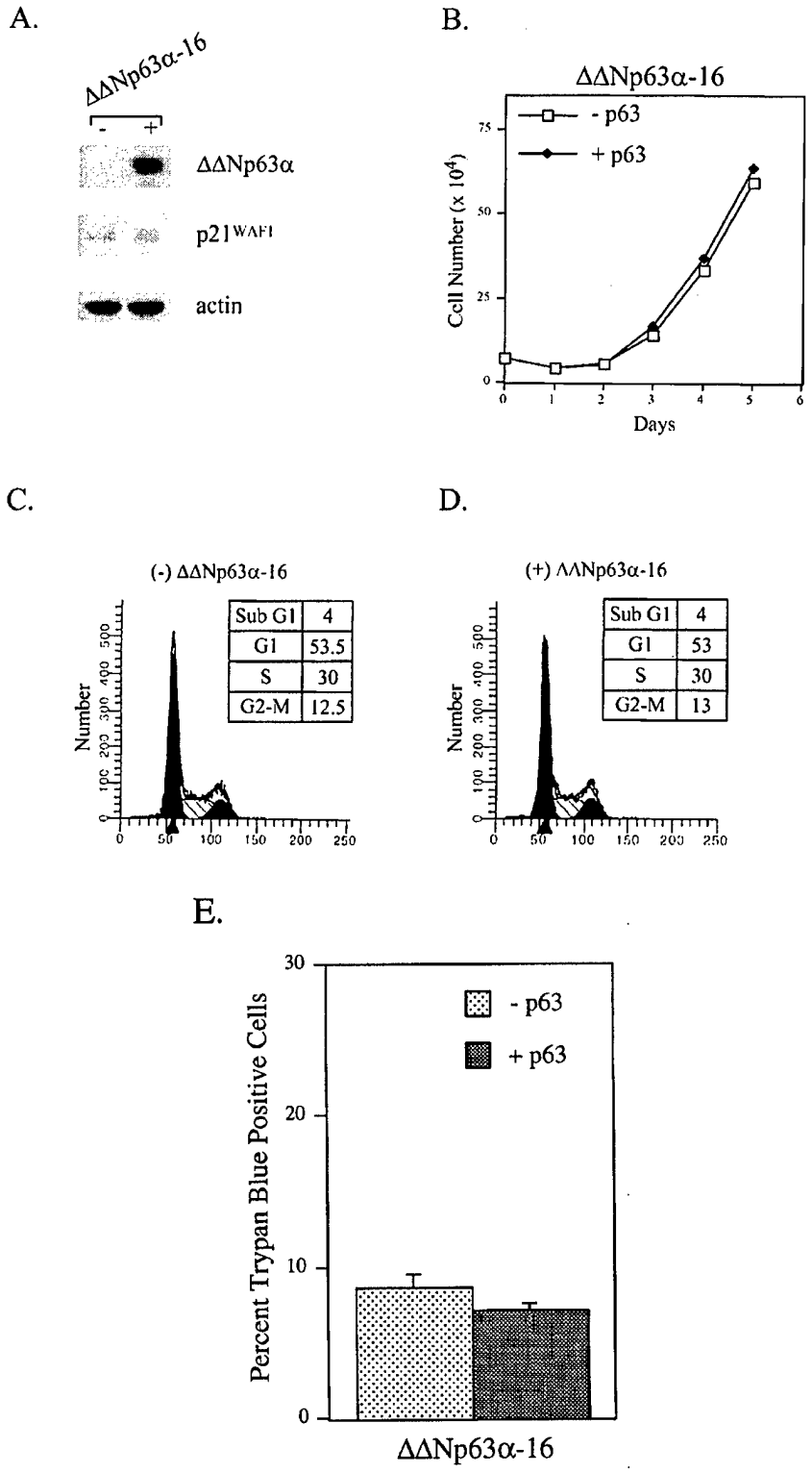

Figure 8 Characterization of ΔΔNp63α-16 cell line, which inducibly expresses a ΔNp63α N-terminal deletion mutant. (**a**) Levels of ΔΔNp63α (upper panel), p21^{WAF1} (middle panel), and actin (lower panel) in the ΔΔNp63α-16 cell line were assayed by Western blot analysis. Cell extracts were prepared from uninduced cells (−) and cells induced (+) to express ΔΔNp63α for 24 h. The blot was probed as in Figure 1. (**b**) Growth rate analysis of ΔΔNp63α-16 cells uninduced (□) or induced (◆) to express ΔΔNp63α as described in Materials and methods. (**c**, **d** and **e**) ΔΔNp63α-16 cells, which were uninduced (−) or induced (+) to express ΔΔNp63α for 3 days, were analysed for DNA content by propidium iodide staining of fixed cells (**c** and **d**) and for percentage of dead cells by Trypan blue dye staining (**e**)

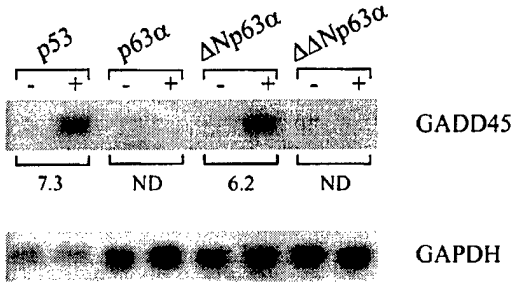

Figure 9 ΔΔNp63α cannot regulate the ΔNp63α target *GADD45*. Northern blots were prepared using 10 μg of total RNA isolated from p53-3, p63α-14, ΔNp63α-22, or ΔΔNp63α-16 cells under both the uninduced (−) and induced (+) conditions for 24 h. The blots were probed with *GADD45* cDNA (upper panel) and *GAPDH* cDNA (lower panel). The fold increase in mRNA expression is shown. *ND*, not done

Discussion

We have described here the characterization of H1299 cell lines that inducibly express p63α and ΔNp63α. We have demonstrated that both p63 isoforms are able to transactivate p53 target genes and induce cell cycle arrest and apoptosis. Deletion of the N-terminal 26 amino acids of ΔNp63α abolishes its ability to transactivate p53 targets and to induce cell cycle arrest and apoptosis, suggesting that a transactivation domain exists within this N-terminal region. Although p63 is homologous to p53 within highly conserved domains, p63 differentially activates some but not all p53 and p73 target genes, indicating that p63 has cellular activities that are both similar to and different from those of the other p53 family members.

Since ΔN isoforms of p63 lack the acidic N-terminus corresponding to the transactivation domain of p53, it has been proposed that these isoforms are transcriptionally inactive and act as dominant negative isoforms (Yang *et al.*, 1998). Here we present evidence that both p63α and ΔNp63α can activate *p21^{WAF1}*, although p63α is a stronger inducer of *p21^{WAF1}* than ΔNp63α (Figure 6a). It is likely that at low doses of a weak transactivator, the purported heterotypic interactions between p63 isoforms may serve to stabilize transactivation-capable variants. At higher doses of a weak transactivator, however, competition for p53 targets and sequestration of transactivating variants will result in an overall decrease in transactivation. This may explain the initial increase in transactivation activity of p63γ when coexpressed with low levels of ΔNp63γ, followed by a decrease in activity with high levels of ΔNp63γ (Yang *et al.*, 1998). Therefore, heterotypic interactions between p63 isoforms may serve to either stabilize or inhibit transactivation activity in a concentration-dependent manner. The effect of heterotypic interactions on transactivation activity appears to be isotype- and target-specific and is underscored by the differential regulation of p53 target genes by p63 isoforms (see Figure 6).

Deletion of the N-terminal 26 amino acids from ΔNp63α produced the mutant ΔΔNp63α, which is incapable of regulating p53 target genes or of inducing cell cycle arrest and apoptosis (see Table 1). This suggests that a second transactivation domain exists within the N-terminus of ΔN isoforms of p63. This is not surprising, as two transactivation domains have previously been found within the p53 N-terminus (Candau *et al.*, 1997; Zhu *et al.*, 1998b). It is highly unlikely that the myc epitope located at the N-termini of these p63 proteins is contributing to this activity, since ΔΔNp63α lacks transactivation activity but retains the myc epitope. Further studies need to be conducted to more precisely map the activation domains of p63.

Our data provide evidence that p63-dependent arrest occurs predominantly in G_1, similarly to that of p53. Both p63α and ΔNp63α were able to transactivate *p21^{WAF1}*, although to a lesser extent than p53 (Figure 6a). The cyclin-dependent kinase inhibitor p21^{WAF1} is known for its role in inducing cell cycle arrest in G_1 (el-Deiry *et al.*, 1993; Harper *et al.*, 1993); however, it remains to be seen whether the weak activation of *p21^{WAF1}* by p63 is sufficient to induce cell cycle arrest or whether additional genes are also involved in p63-mediated cell cycle arrest.

p53-dependent G_2-M arrest is mediated in part by the DNA-damage inducible genes *GADD45*, *BTG2*, and *14-3-3σ* (Hermeking *et al.*, 1997; Kastan *et al.*, 1992; Rouault *et al.*, 1996). GADD45 has been shown to bind proliferating cell nuclear antigen (Smith *et al.*, 1994) and induce G_2-M arrest in part by inhibiting Cdc2 protein kinase activity (Jin *et al.*, 2000; Zhan *et al.*, 1999). As shown in Figure 6c, *GADD45* was highly induced by ΔNp63α but not by p63α. The predominant G_1 arrest observed during p63 expression (see Figure 4) may limit the number of cells entering the G_2-M phase, thus accounting for the lack of substantial increase in G_2-M cells during ΔNp63α expression. Furthermore, *BTG2* and *14-3-3σ*, two genes implicated in G_2-M arrest (Chan *et al.*, 1999; Rouault *et al.*, 1996), were not induced by either p63α or ΔNp63α (data not shown). This lack of induction of *BTG2* and *14-3-3σ* correlates with the absence of a predominant p63-dependent G_2-M arrest in H1299 cells.

It has previously been reported that p63α and ΔNp63α failed to transactivate the minimal p53-binding sequence *PG-13* (Kern *et al.*, 1992) within a β-galactosidase reporter construct (Yang *et al.*, 1998). This is not surprising and is consistent with our observation that p63α and ΔNp63α can induce some but not all endogenous p53 target genes (see Figure 6). In addition, it is well established that the activation of transfected promoter constructs does not always reflect the activation of endogenous genes where chromatin remodeling occurs (Smith and Hager, 1997). The chromatin structure of these endogenous p53 target genes may affect p63-mediated transcription.

However, the induction of *GADD45* by ΔNp63α, but not by p63α, is surprising. This disparity in transactivation indicates that the N-terminal domain absent in the

ΔNp63α isoform may inhibit p63α-mediated activation of certain genes. The N-terminus of p63α may confer steric hindrance such that it cannot bind and/or activate the *GADD45* promoter. Perhaps interactions between the N-terminus of p63α and additional proteins reduce the transcriptional activity of p63. p53, p73, and KET, the rat homolog of p63α, have been shown to interact with Wilms Tumor 1 (WT1) protein (Maheswaran *et al.*, 1993; Scharnhorst *et al.*, 2000), and WT1 can negatively modulate the transcriptional activity of both p53 and p73 (Maheswaran *et al.*, 1993, 1995; Scharnhorst *et al.*, 2000). It is likely that WT1 can also inhibit the activity of p63α to transactivate *GADD45*. Although the binding domain(s) of WT1 on p53 family members is not known, the truncated N-termini of ΔNp63 variants may prevent interaction with, and regulation by, WT1 or other proteins that may modulate p63 activity.

p63α and ΔNp63α have been reported to be incapable of inducing apoptosis in transient transfection assays (Yang *et al.*, 1998). Because these assays were conducted with transiently transfected cells, the long-term effects of p63α and ΔNp63α activity were not detectable. As shown in Figure 3, noticeable cell death did not occur until 2–3 days post-induction of p63α and ΔNp63α. This suggests that although the p63γ isoform can induce apoptosis within 16 h (Yang *et al.*, 1998), p63α- and ΔNp63α-mediated apoptosis is a late occurring event. This is supported by the lack of p73α- and p73β-mediated apoptosis in identical transient transfection assays (Yang *et al.*, 1998), whereas long-term expression of these p73 isoforms results in profound cell death (Zhu *et al.*, 1998a).

Although both p63α and ΔNp63α can induce apoptosis, the differential regulation of p53 target genes indicates that the signaling pathways that lead to apoptosis differ from that of p53. Several genes involved in p53-dependent apoptosis (*PIG6*, *PIG7* and *PIG11*) were not significantly induced by p63α or ΔNp63α (data not shown), whereas *PIG3* was strongly induced by p63α (Figure 6f). *PIG8*, which has been shown to induce apoptosis (Gu *et al.*, 2000), was weakly induced by p63α but not by ΔNp63α (Figure 6g). *p85*, an apoptotic signal transducer in the cellular response to oxidative stress (Yin *et al.*, 1998), was not induced by p63α or ΔNp63α (data not shown). The DNA-damage inducible *KILLER/DR5* gene may mediate p53-dependent apoptosis (Wu *et al.*, 1997), and it was weakly induced by ΔNp63α, but not by p63α (Figure 6e)

MDM2, an oncogene that negatively regulates p53 activity (Wu *et al.*, 1993), was weakly activated by p63α but not by ΔNp63α (Figure 6b). Physical interaction between MDM2 and the N-terminus of p53 inhibits the transactivation activity of p53 and enhances the degradation of p53 through the ubiquitination pathway (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997; Oliner *et al.*, 1993). Although it is unclear whether p63 physically interacts with MDM2, activation of the oncogene by p63α suggests that this isoform is regulated by a similar mechanism as p53. The N-terminal truncation found in the ΔN isoforms may prevent interaction with MDM2. In addition, *MDM2* was not activated by ΔNp63α. Therefore, the predominant expression of ΔN isoforms in epithelial tissues (Crook *et al.*, 2000; De Laurenzi *et al.*, 2000; Hall *et al.*, 2000; Yang *et al.*, 1998) may result from the lack of negative regulation by MDM2.

As shown in Figure 2, the p63-expressing cell lines express lower levels of p63 than the ME-180 cell line. However, in contrast to H1299 cells, proliferation of ME-180 cells does not appear to be inhibited by p63. This may be due to genetic aberrations within the ME-180 cell line that render the cells insensitive to p63. The presence of human papilloma virus (HPV) DNA in the ME-180 cell line suggests that disruption of the pRb and p53 pathways by E7 and E6 proteins (Boyer *et al.*, 1996; Scheffner *et al.*, 1993), respectively, may serve to immortalize these cells and diminish the anti-proliferative activity of p63 (for reviews, see Barbosa, 1996; zur Hausen, 2000). Furthermore, attempts to clone p63 from ME-180 cells have yielded cDNAs with mutations resulting in an altered amino acid composition that could potentially affect p63 activity (data not shown).

Our data indicate that p63α and, more importantly, ΔNp63α are transcriptionally active and can induce cell cycle arrest and apoptosis. Our results emphasize the need to reassess the activities and functions of ΔN variants of p63 and possibly of p73. It is not yet determined whether p63 is a true tumor suppressor like its sibling p53; however, the ability of p63 isoforms to differentially regulate p53 target genes and execute similar functions suggests that at least some signaling pathways are utilized by all members of the p53 family.

Materials and methods

Plasmids and mutagenesis

cDNAs from p63α and ΔNp63α (kindly provided by C DiComo and C Prives) were cloned separately into a tetracycline-regulated expression vector, pUHD10-3, at its *Bam*HI site, and the resulting plasmids were used to generate cell lines that inducibly express p63. p63 proteins were tagged at their N-termini with a myc epitope. The mutant ΔΔNp63α construct (see Table 1) was generated by PCR. The ΔNp63α cDNA was amplified by a 5 primer (5′-GAT CGG ATC CAC CAT GGG CGA GCA GAA GCT CAT CTC AGA AGA AGA CCT CGA AGA CCA GCA GAT TCA G-3′) and by a 3 primer (5′-GTC TGT TCA TTC CTC CGA CGC A-3′) designed to incorporate a myc-tag at the N-terminus and eliminate the first 26 codons of ΔNp63α. The resulting cDNA fragment was used to replace the 5′ region of ΔNp63α between the *Bam*HI and *Eco*RI sites.

Cell culture and cell lines

H1299 is a p53-null non-small cell lung carcinoma cell line. Cells were cultured in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum. H1299 cell lines that inducibly

express p63 were generated as described previously (Chen *et al.*, 1996). Individual clones were screened for inducible expression of p63 protein by Western blot analysis using anti-myc epitope monoclonal antibody 9E10.2. The H1299 cell lines that inducibly express wild-type p53, p73α, and p73β are p53-3, p73α-22, and p73β-9, respectively, as described previously (Chen *et al.*, 1996; Zhu *et al.*, 1998a). The human cervical carcinoma cell line ME-180 was purchased from American Type Culture Collection (Rockville, MD, USA).

Western blot analysis

Cells were washed and collected from plates in phosphate-buffered saline (PBS) solution, resuspended with 2 × sample buffer and boiled for 5 min. Western blot analysis was performed as described previously (Chen *et al.*, 1996). Affinity purified anti-actin polyclonal antibody was purchased from Sigma Chemical Co. (St. Louis, MO, USA). 9E10.2 was purchased from American Type Culture Collection (Rockville, MD, USA). Anti-p21 antibody C19 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p63 monoclonal antibody Ab-1 was purchased from Oncogene Research Products (Cambridge, MA, USA).

Growth rate analysis

Cells were seeded at approximately 7.0×10^4 cells/60-mm plate with or without tetracycline (1.0 μg/ml). The medium was replaced every 72 h. At the times indicated, two plates were rinsed with PBS twice to remove dead cells and debris. Live cells on the plates were trypsinized and collected separately. Cells from each plate were counted four times using a Coulter cell counter, and the average number of cells from both plates was used for growth rate determination.

FACS analysis

Cells were seeded at 2.0×10^5/90-mm plate, with or without tetracycline (1.0 μg/ml). Three days after plating, both floating dead cells in the medium and live cells on the plate were collected and fixed with 1 ml of 75% ethanol for at least 1 h at 4°C. The fixed cells were centrifuged and resuspended in 0.5 ml PBS solution containing 20 μg/ml of RNase A (Sigma Chemical Co., St. Louis, MO, USA) and 50 μg/ml of propidium iodide (Sigma Chemical Co., St. Louis, MO, USA). The stained cells were analysed in a fluorescence-activated cell sorter (FACSCaliber; Becton Dickinson, Menlo Park, CA, USA) within 4 h. The percentage of apoptotic cells containing a sub-G_1 DNA content was quantified using the CellQuest program. The percentage of live cells in the G_0–G_1, S, and G_2-M phases was quantified using the ModFit program.

Annexin V staining assay

The annexin V staining assay for apoptosis is based on the ability of annexin V to bind to phosphatidylserine. In healthy cells, phosphatidylserine is located on the inner leaflet of the plasma membrane (Vermes *et al.*, 1995). Upon induction of apoptosis, phosphatidylserine can flip to the outer surface of the plasma membrane and bind annexin V (Vermes *et al.*, 1995), which is labeled with a fluorescent dye and can be detected by FACS analysis. Both dead and live cells were collected and washed twice with cold PBS. The cells were resuspended in 0.1 ml of annexin V binding buffer to a density of 1×10^6/ml and stained according to the manufacturer's instructions (Boehringer Mannheim, Germany).

Trypan blue dye exclusion

Trypan blue dye staining is based on the principle that certain dyes will not stain live, viable cells, whereas dead, unviable cells are susceptible to staining. Cells were seeded at approximately 7.0×10^4 cells/60-mm plate with or without tetracycline (1.0 μg/ml). Three days after plating, live and dead cells from two plates were collected separately and mixed with an equal volume of 0.4% Trypan blue dye solution (Sigma Chemical Co., St. Louis, MO, USA) for 15 min. Stained (dead) and unstained (live) cells were counted using a hemocytometer and the percentage of dead cells/total cells was determined by scoring an average of over 300 cells, twice per plate.

Mitochondrial membrane assay

Cells were seeded at 2.0×10^5/90-mm plate, with or without tetracycline (1.0 μg/ml). Three days after plating, both floating dead cells in the medium and live cells on the plate were collected and stained with MitoSensor reagent (Clontech, Palo Alto, CA, USA) for 20 min at 37°C before analysis by flow cytometry.

Luciferase assay

The *p21^{WAF1}* promoter was cloned upstream of a luciferase reporter gene (Chinery *et al.*, 1997). The p53 response element found within the *EphA2* promoter (Dohn *et al.*, 2001, unpublished results) was cloned upstream of a minimal c-*fos* promoter and a firefly luciferase gene (Johansen and Prywes, 1994). 1.0 μg of the resulting reporter vectors was cotransfected into H1299 cells with 2.0 μg of pcDNA3 control vector or a vector expressing p53, p63α, or ΔNp63α. For an internal control, 25 ng of the Renilla luciferase vector pRL-CMV (Promgea, Madison, WI, USA) were cotransfected with the above constructs. Transfections were performed as described (Chen and Okayama, 1987), and dual luciferase assays were performed in triplicate according to the manufacturer's instructions (Promega, Madison, WI, USA).

RNA isolation and Northern blot analysis

Total RNA was isolated from cells using Trizol reagents (Life Technologies, Inc., Gathersburg, MD, USA). Northern blot analysis was performed as described (Zhu *et al.*, 1998b). The *p21^{WAF1}*, *MDM2*, *BAX*, *GADD45*, and *GAPDH* probes were prepared as described previously (Zhu *et al.*, 1998b). The *KILLER/DR5* cDNA probe (GenBank #159553) was purchased from American Type Culture Collection (Rockville, MD, USA). The following cDNA probes were purchased from Genome Systems, Inc. (St. Louis, MO, USA): *BTG2* (GenBank #H86711), *14-3-3σ* (W79136), *PIG2* (H18355), *PIG3*(N75824), *PIG6* (R88591), *PIG7* (H98066), *PIG8* (R42786), *PIG11* (R54648), and *p85* (N21330).

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